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# **Studies In Lactic Acid Fermentation**

BY

**JAMES M. NEILL**

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**PART I**

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"STUDIES ON LACTIC ACID FERMENTATION".

James M. Neill

Thesis Submitted for the Degree of Doctor of Philosophy.

Massachusetts Agricultural College

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## "STUDIES ON LACTIC ACID FERMENTATION".

This thesis involves three distinct studies on lactic acid fermentation, a process of great importance in agriculture and in other sciences. These studies, while distinct entities, are intimately related. Each study represents the pursuit of a definite object or purpose; the attainment of the object of each of the studies represents a contribution to questions involved in the purposes of the other parts of the thesis.

The purposes of the thesis are the basis of its division into the following three parts:

### Part I.

#### "A REVIEW OF THE LITERATURE OF LACTIC ACID FERMENTATION".

The "Review of the Literature of Lactic Acid Fermentation" represents an attempt to collect and systematize the extensive literature which has been contributed in the various fields in which this fermentation assumes importance.

In this review, a number of interesting and important questions become evident, and furnish the basis of the remainder of the thesis.

### Part II.

#### "A STUDY OF THE CHARACTERS OF THE STREPTOCOCCI OF DAIRY LACTIC ACID FERMENTATIONS, WITH SPECIAL REFERENCE TO THE PRESENT STATUS OF THE SO CALLED STREPTOCOCCUS LACTICUS GROUP".

The "Study of the Characters of the Streptococci of Dairy Lactic Acid Fermentations" consists of an investigation of the value of the different characters which have been used to describe the lactic streptococci of sour milk. This group of



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microorganisms includes lactic acid bacteria which are of peculiar importance in agricultural lactic fermentation. The relation of this part of the thesis to Part I is evident.

### Part III.

"A COMPARATIVE STUDY OF DIFFERENT TYPES OF STREPTOCOCCI, WITH SPECIAL REFERENCE TO THE PEPTOLYTIC ACTIVITY OF THE LACTIC GROUP".

The "Comparative Study of Different Types of Streptococci" has to do with an intimate comparison of the lactic streptococci with other types of streptococci. These other types of streptococci are also lactic acid bacteria, and are closely related to the lactic type of sour milk. Thus, this study is directly related to Parts I and II.

Part III is divided into two sections, upon the basis of the following purposes of this part of the thesis:

#### Section A.

"A Comparison of the Relative Influence of Environmental Conditions upon the Life Processes of Different Types of Streptococci".

#### Section B.

"A Comparison of the Peptolytic Activity of Different Types of Streptococci, with Special Reference to the Lactic Group".

PART I.

"A REVIEW OF THE LITERATURE  
OF LACTIC ACID FERMENTATION"

The following statement of the purpose, scope, and general method of presentation of this part of the thesis are offered at this place, not as an introduction, but rather as an explanation of certain questions that may arise in the reader's mind upon analysis of the text of the manuscript.

This review of the literature of lactic acid fermentation was suggested to me by Dr. Marshall, in the winter of 1918. It was begun at that time with the object of collecting the original literature on this fermentation and making it accessible to students in agricultural and general microbiology. In the collection of material, and in its arrangement, the attempt has been made to fit the review for use as a reference source for the student in general microbiology, and also to a certain extent for the advanced student in special fields in which lactic acid fermentation assumes importance.

This review has attempted to cover in a fairly complete manner the literature on lactic acid fermentation. Due to the extensive boundaries of this fermentation process, it has been necessary to limit the fields covered in this paper. The attempt has been made to concentrate the review upon the general fundamentals of the process of the production of lactic acid by microorganisms. The practical applications of the reaction are suggested rather than defined. In addition to the omission of definite discussions of the industrial application of the fermentation, the following subjects are omitted. The sanitary and hygienic relations of the colon-typhoid group of bacteria have led to the development of a definite literature field, in which the lactic fermentation relations of these bacteria are important. The question of the use of fermented milks as therapeutic or prophylactic agents has also led to an extensive literature in which, again, other questions dominate those involved in the fermentation itself. (This field has been reviewed by a number of authors, recently by Rettger and Chaplin.) The literature on the production of lactic acid by animal tissue, and the rôle of lactic acid in animal metabolism and in general animal physiology, are not included in this review.



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All of these are fields which are more or less specialized and which have been reviewed by competent authorities.

Under the general fundamentals of lactic acid fermentation by microorganisms, have been included the history and chemistry of the lactic acid fermentation reaction, the lactic acid bacteria as the microbial agents of the process, the influence of the environment upon the agents of the fermentation, and the products of the reaction. Under the last mentioned topic, considerable emphasis has been put upon the chemistry of lactic acid itself, as a means of emphasizing to the student that microbial lactic acid fermentation is essentially a chemical reaction in which at least the more prominent products are clearly defined chemical substances.

The general method of presentation of the text requires the following explanations. A general outline of the subject is presented in which the review is divided into nine headings or chapters. The whole review is then presented with the attempt to make each of these chapters a more or less independent link in the logical development of the subject as a whole. Then, for each of the chapters a detailed analysis or outline is presented. All of the material in the literature which would be included in each chapter is presented under the different headings and subheadings of the chapter analysis. As a result, the same article may be reviewed in a number of the different chapters, but from different aspects. It seemed that this method was to be preferred to the introduction of a system of cross references.

In keeping with the attempt to maintain the integrity of the individual chapters, the references quoted in the text are presented at the end of each chapter as authority for the statements made. The author-subject bibliography is presented at the end as a separate division. It includes practically 900 published articles on lactic acid and lactic acid fermentation. Of these, six hundred are quoted in the text. These represent references obtained before May 1, 1931, the time of completion of the text manuscript. The rest of the bibliography consists of references obtained during the past year. Unless marked otherwise, these references have been reviewed and material indexed for inclusion in the text if a revised copy is desired at a future date. No

attempt has been made to comb the literature for an extensive bibliography of merely cited references, and the literature has been thoroughly reviewed only from 1911 to 1921. Collections of much of the older literature of the period from 1890 to 1910 have been made in various German handbooks and in a number of "Jahresberichte". Little would be gained by the inclusion of these references unless of sufficient importance to be actually reviewed in the text. Considerable work has been spent in obtaining original articles of historical interest.

In connection with this review of Part I of my thesis, I wish to express my appreciation to the following: to Dr. C. E. Marshall, for the initial suggestion of the work and for his careful and painstaking review of the complete manuscript; to Mr. Conrad H. Lieber, for the drawing of all of the charts and figures; to Mr. Charles Green, for his courtesy in obtaining a number of references not accessible in the library.

# REVIEW OF THE LITERATURE OF LACTIC ACID FERMENTATION

## General Outline

- A. HISTORY OF DEVELOPMENT OF INTERPRETATION OF  
LACTIC ACID FERMENTATION
- B. CHEMICAL CHANGES INVOLVED IN LACTIC ACID  
FERMENTATION
  - I. Transformation of Matter
  - II. Transformation of Energy
- C. THE LACTIC ACID BACTERIA
- D. ENZYMES OF THE LACTIC ACID BACTERIA
- E. INFLUENCE OF ENVIRONMENT UPON LACTIC ACID BACTERIA
  - I. Physical Influences
  - II. Biochemical Influences
  - III. Intermicrobial Influences
- F. PHYSIOLOGICAL EFFICIENCY OR FERMENTING CAPACITY  
OF LACTIC ACID BACTERIA
- G. THEORETICAL PROGRESS OF LACTIC ACID FERMENTATION
- H. PRINCIPAL PRODUCT OF LACTIC ACID FERMENTATION
  - I. Lactic Acid as a Chemical Substance
  - II. Stereochemical Lactic Acid Fermentation
  - III. Amount of Lactic Acid Formed
- I. OTHER PRODUCTS OF LACTIC ACID FERMENTATION
- J. BIBLIOGRAPHY



A. HISTORY OF DEVELOPMENT OF INTERPRETATION OF LACTIC  
ACID FERMENTATION.

# HISTORY OF DEVELOPMENT OF INTERPRETATION OF LACTIC ACID FERMENTATION

- I. Earliest Observations of Lactic Acid Fermentation.
- II. Chemical Interpretation.
  1. Pelouze and Gay Lussac.
  2. Fremy and Boutron Chalard.
- III. Microbial Interpretation.
  3. Difficulties encountered.
  4. Blondeau's conjecture.
  5. Pasteur's contribution.
  6. Lister's work.
- IV. Isolation of Lactic Acid Bacteria as Agents of Important Lactic Acid Fermentations.
  1. Alcoholic fermentation industries.
  2. Fermentation plant products.

## HISTORY OF DEVELOPMENT OF INTERPRETATION OF LACTIC ACID FERMENTATION\*

### I. Earliest Observations of Lactic Acid Fermentation.

Lactic acid fermentation was probably the first fermentation process to be observed by man. In the earlier nomadic life, the alteration and souring of the milk of their flocks upon standing in the crude vessels and bags must have puzzled the primitive races. Since all other fermentations would demand a more settled state of living and the attainment of a higher degree of skill, it may be assumed that lactic acid fermentation was the first fermentation process entering into the domestic economy of man. (Lafar, p. 170)

In spite of the fact that lactic acid fermentation was probably the first fermentation process to be brought to man's attention, it was one of the last fermentations to be correctly interpreted. It was 1780 before the chief product of the process was discovered and not until much later was it recognized as the product of a particular fermentation.

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\* Naturally, a complete discussion of the early history of our knowledge of lactic acid fermentation would be concerned most intimately with the controversy over the theory of spontaneous generation, and with the establishment of the germ theory of fermentation. Such a treatment is beyond the scope of this paper.



During the period when so many investigators were using the microscope in examining various substances for the presence of "infusoria", Anđry (in 1701) reported an observation of living organisms in sour milk. This investigation, however, was unproductive.

## II. Chemical Interpretation.

The fermentation theories of Caignard Latour and Kützing were not soon applied to lactic acid fermentation, and for a long time the purely chemical theories dominated in the interpretation of this process. In 1833 Pelouze and Gay-Lussac carried out the first complete investigation of the souring of milk. Fremy and Boutron carried out experiments upon the action of animal membranes upon sugar solutions. As a result of this work, under the influence of Liebig's fermentation theory, they concluded that the albumin substance of the animal membrane acted as the "ferment" which produced the changes in the media.

From later experiments Fremy and Boutron-Chalard (1841) came to the conclusion that this action was not confined to animal membranes alone. "All the organized matter of vegetable or animal origin, after being exposed to the air for some time, are able to change neutral substances into lactic

acid." They carried out experiments on the formation of lactic acid from different sources, those particularly of interest here being the lactic acid fermentations of vegetables and of milk. In the latter case, they believed casein\* to be the "true ferment of the milk sugar, or at least that casein is to milk sugar, what beer yeast is to cane sugar." They recognized the fact that their "ferments" could cause the production of other substances than lactic acid, but attributed that to a successive alteration of the ferment. In spite of their chemical interpretation, their conclusions mark a step in advance in that they believed "that this acid (lactic acid) is not merely one of the products of a complex fermentation, but that it is indeed the result of a special ferment to which we give the name 'fermentation lactique'."

Rowlandson, 1852, under the influence of the Liebig, and especially of the Gay-Lussac theory, defined the conversion of lactose into lactic acid as an oxidation process. He went so far as to claim that milk obtained from a cow that had been running about and, therefore breathing rapidly, would sour with unusual rapidity.

The chemical theories were much strengthened in their application to lactic acid fermentation,

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\* As late as 1889, Fokker attempted to establish that casein was the cause of lactic acid fermentation of milk.

due to the difficulty of rendering milk sterile, as experienced by Schröder and von Dusch, (1854, 1859), when this medium was used by the opponents of the theory of spontaneous generation.

### III. Microbial Interpretation.

The interpretation of lactic acid fermentation as being due to the vital activity of micro-organisms was delayed because of the reasons mentioned, and still further because the micro-organisms concerned were not so evident as the "mother" of vinegar, the yeast of beer, and the "infusoria" of putrefaction and decay. Long after the observations of Andry mentioned above, Blondeau, 1847, observed micro-organisms in sour milk. Neither of the two types he distinguished <sup>is</sup> are concerned in lactic acid fermentation, although he considered one of them as an agent of that process. Turpin's (1837) investigation of the bacteria in milk did not yield any knowledge of the lactic acid bacteria.

Somewhat later, a working basis was established for the proof of the role of micro-organisms in lactic acid fermentation, by the researches of Pasteur, who rendered milk sterile by heating it above 100°.

He proved that lactic acid fermentation is the result of the action of micro-organisms, by inducing that fermentation in sterile milk by inoculation with his lactic "ferment". His work also



established that lactic acid fermentation is a specific process, distinct from alcoholic and other fermentations, by proving that his lactic acid "yeast" always set up lactic acid fermentation in sugar media, while the alcoholic "ferment", under like conditions, caused alcoholic fermentation. This marks the establishment of the applicability to lactic acid fermentation of Kützing's theory of specific ferments; it was proof that lactic acid fermentation is due to the life processes of certain micro-organisms.

The observations of Boutroux, Piroetta, and Vandevelde, on the bacteria responsible for this fermentation were of but little value, as their organisms were not lactic acid bacteria.

#### IV. Isolation of Lactic Acid Bacteria as Agents of Important Lactic Acid Fermentations.

It remained to be established just what particular micro-organisms acted as agents of lactic acid fermentation. Without detracting in the least from the value of Pasteur's work, it is hardly possible that his lactic "yeast" was a pure culture, as no means of isolation of species had been attained in bacteriologic technique.

Lister, 1873, had obtained an organism from sour milk, which, when inoculated into sterile milk, produced lactic acid, but the organism was probably a mixture of many species. Later, 1878, he published a description of a bacterium isolated from sour milk by his dilution method. Possibly this was a pure culture.

Huepft, 1884, by use of Koch's plate method, isolated from sour milk the first lactic organism known definitely to be a pure culture. In his description of this organism, he proposed the name *B. acidi lactici*. He claimed it to be the usual agent of lactic acid fermentation in milk. The acceptance of this assumption by many investigators had rather a harmful effect on the early microbial interpretation of lactic acid fermentation, as it led not only to a confusion in the nomenclature of lactic acid bacteria, but led also to a faulty conception of the lactic acid fermentation process itself. Huepft's organism is probably identical with the *B. lactis aerogenes* isolated by Escherich (1885) from the stools of an infant.\*

Leichmann, in 1894, Günther and Thierfelder, in 1895. Eten, in 1896, possibly also

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\* See "Acid Gas Group" under Lactic Acid Bacteria.

Beyer, in 1886. (ref. Esten), were more fortunate in the organisms they isolated. Günther and Thierfelder, and also Esten, although it is evident from their descriptions that their organisms were distinctly different from *B. acidilactici*, believed them identical with Hueppe's bacillus. Leichmann, however, distinguished his culture from that of Hueppe and named it, perhaps unwisely, *B. lactis acidii*\*\*. This was a distinct step in advance --- there had now been obtained in pure culture the most common agent of agricultural lactic acid fermentation. Following this, a great number of species of lactic acid bacteria were isolated (ref. to literature: Lafar, Emmerling). The work of Conn and Esten stands out prominently in the later part of this period.

Probably the most valuable of the early work in lactic acid fermentation was done in investigations of natural fermentations of milk. Further research, yielding definite knowledge concerning the lactic acid fermentation of various milk

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\*\* Probably identical with *Strep. lactis* Kruse. See "*Strep. Lacticus* Group" under Lactic Acid Bacteria.

products soon followed. Investigations in the alcoholic fermentations industries were further advanced and contributed much valuable knowledge concerning the lactic acid bacteria found in the brewery and distillery. Marpmann, in 1886, had studied lactic acid fermentation of fermented plant products, but not until several years after the establishment of the lactic acid fermentation of milk, were the lactic fermentations of these products extensively studied by Conrad (1897), Aderhold (1899), and others.



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D. CHEMICAL CHANGES INVOLVED IN LACTIC ACID  
FERMENTATION.

## CHEMICAL CHANGES INVOLVED IN LACTIC ACID FERMENTATION

### I. Transformation of Matter.

1. General discussion of changes involved.
  - a. Empirical formulae.
  - b. Division of lactic acid fermentations upon chemical basis.
2. Changes involved in "true" lactic acid fermentation.
  - a. Hexoses.
    - (1) Temporary entrance of water molecules.
    - (2) Migration of atoms.
  - b. Polysaccharoses (yielding hexoses).
3. Changes involved in "mixed" lactic acid fermentations.
  - a. "Acid gas" fermentation of hexoses.
    - (1) Structural explanations.
    - (2) Simultaneous reactions.
  - b. Other "mixed" lactic acid fermentations of hexoses.
  - c. "Acid gas" fermentation of mannitol.
  - d. Fermentation of pentoses.
4. Rôle of intermediate substances.
  - a. Introduction.
  - b. Bayer's anhydride theory.
  - c. Trices as probable intermediate substances.
  - d. Intermediate substances in "mixed" lactic acid fermentations.



## II. Energy Changes Involved in Lactic Acid Fermentation.

1. Thermodynamics of lactic acid fermentation.
  - a. Source of energy required.
  - b. Energy yielded in lactic acid fermentation.
2. Chemical nature of the fermentation process.
  - a. Rearrangement of matter and of energy involved in the reaction.
  - b. Comparison of oxidation and fermentation.
    - (1) "Intramolecular oxidation."

Simultaneous oxidation and reduction.  
Part of energy absorbed in products.
    - (2) Degree of completeness of energy transformation.
3. Biological nature of the fermentation process.
  - a. Physiological significance of energy change of lactic acid fermentation reaction.
  - b. Relation of oxygen to life.
  - c. Comparison of fermentation and respiration.
  - d. Biological significance of the degree of energy transformed.

## CHEMICAL CHANGES INVOLVED IN LACTIC ACID FERMENTATION

### I. Transformation of Matter.

#### 1. General discussion of changes involved.

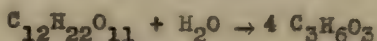
Lactic acid fermentation is a biochemical reaction, by which various organic compounds, usually carbohydrates, are transformed, at least in part, to lactic acid.

The lactic fermentation of sugars involves an internal rearrangement of the molecule, as in alcoholic fermentation. Upon first examination, this change appears very simple and seems to be represented by the equation,

(1) in case of hexoses,



(2) in case of disaccharoses,



The changes involved are, however, not so simple; part of the fermented substance seems always to be diverted to the use of the cell; other products may be formed; the exact nature and course of the reactions are not established. Moreover, even when lactic acid is almost the only product, the different sugars may be split differently and the fermentation may yield different products, according to the lactic

micro-organisms, nourishment and environmental conditions, and time of analysis.

A discussion of the chemistry of the changes involved may be divided as follows:--

"True" lactic acid fermentation of { hexoses  
polysaccharoses

in which lactic acid is almost the only product.

"Mixed" lactic acid fermentation of { pentoses  
hexoses  
polysaccharoses

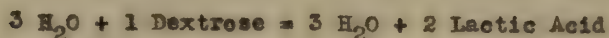
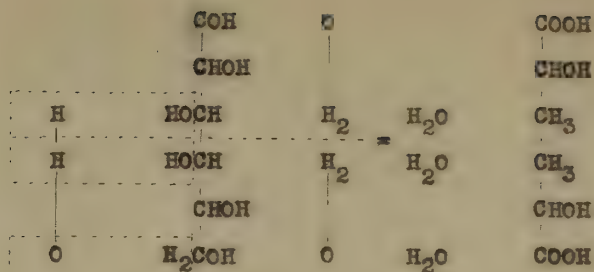
in which other products arise in appreciable quantities.

2. Changes involved in "true" lactic acid fermentation.

### a. Hexoses.

Various hypotheses have been advanced to explain the structural changes involved in the empirical formulas of the lactic acid fermentation reaction in which lactic acid is the only appreciable product. Duclaux (1901) proposed a structural explanation of the molecular disintegration of a hexose molecule into two molecules of lactic acid as an exchange of atoms between the carbon groups of the sugar molecule.

Jensen(1909) advanced a somewhat different explanation, in which water enters into the reaction but does not affect the products; the real change is explained as a rearrangement of oxygen and hydrogen atoms.



Many other authorities, (among them, Kruse and Oppenheimer), believe that the "splitting" or rearrangement of the hexose molecule involves a temporary entrance of a H<sub>2</sub>O molecule which is set free with formation of lactic acid.\*

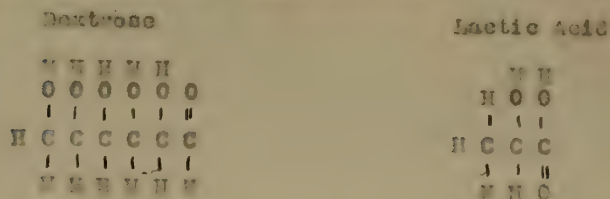
Although no oxygen molecules enter into the equations of the reactions occurring in these so-called "true" lactic acid fermentations, Rahn (1917) and others believe that reactions of oxygen atoms, within the sugar molecule, are involved in changes by which one side of the molecule is oxidized and the other side reduced.\*\* By this internal oxidation, the oxygen, which is distributed evenly among the carbon groups of the sugar molecule, is shifted to one side of the molecule in the lactic acid produced. This relation is readily seen

\* See references under "Intermediate Substances."

\*\* See "Energy Transformation."



by a comparison of the structural Formulae of each.



Stoklasa (1904) uses the term "glycolyse" to represent such rearrangement of oxygen atoms within the molecule. All explanations of the chemical changes involved in lactic acid fermentation are based, to a certain extent, upon intramolecular migration of oxygen atoms.

It is evident that there is a migration of H atoms as well as of O atoms; it also follows that, if there is intramolecular oxidation, there must also be intramolecular reduction. In recent years, the rearrangement of H atoms and intramolecular reductions have been interpreted by a number of authorities, as being of equal importance to the accompanying intramolecular oxidations.

In the case of alcoholic fermentation, reduction reactions have been emphasized particularly by a recent group of investigators. Bailey and Baldwin (1916) point out that the "anaerobic" oxidations involved in alcoholic fermentation are dependent upon reduction reactions in the same system.

In Gray's interpretation of acid gas lactic acid fermentation (1916, 1920), hydrogen

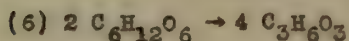
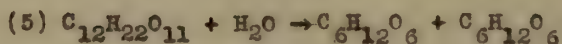
atoms and their reducing action upon intermediate products, assume the greatest importance. (See "Role of Intermediate Substances" under "Chemical Changes", and "Other Products of Lactic Acid Fermentation".)

b. Polysaccharoses (yielding hexoses).

The preceding explanations have been concerned with the chemical changes which a hexose undergoes to form lactic acid. However, as will be shown later, lactic acid fermentation is by no means confined to hexoses. Of the many other carbohydrates which undergo "true" lactic acid fermentation, we will consider the disaccharoses, such as lactose, sucrose and maltose.

In most textbooks it is assumed that the production of lactic acid from a disaccharose is always preceded by a hydrolysis, which yields two hexose molecules, and that the lactic acid itself is produced by the splitting of the hexose compo-

nents. The order of reactions is held to be as follows:--



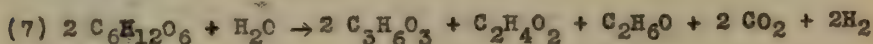
Whether hydrolysis precedes the lactic acid fermentation of the higher sugars, or whether they are changed more directly to lactic acid, is a disputed question. It is known that a disaccharose must first be hydrolyzed before it undergoes alcoholic fermentation, and as lactic acid fermentation is similar in so many respects to alcoholic fermentation, many assume that the same sequence of reactions takes place here. However, it will be shown in the discussion of "Enzymes" (see p. <sup>III, l. a. "Enzymes"</sup>) that it is not yet definitely proven that higher carbohydrates are, in all lactic acid fermentations, first hydrolyzed into their simple hexose constituents before they undergo lactic acid fermentation.

The discussion and equations given above are applicable only to those lactic acid fermentations, in which the disintegration of the sugar yields at least almost nothing but lactic acid, as in the "true" lactic acid fermentation of Duclaux and Kayser.

### 3. Changes involved in "mixed" lactic acid fermentations.

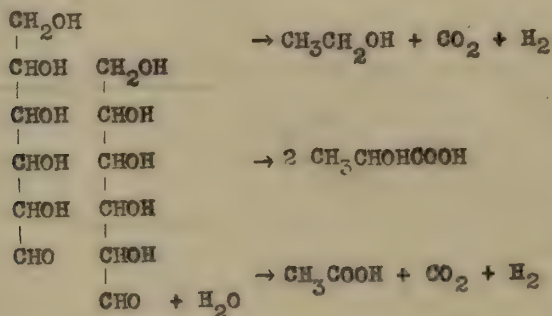
#### a. Acid gas fermentation of hexoses.

The explanation of the chemical changes involved in lactic acid fermentations, in which other products arise in appreciable quantities, is even more difficult than in the fermentations just considered. Harden (1901) proposed the following equation as a representation of the chemical changes involved in the acid gas lactic acid fermentation of glucose or of fructose by *B. coli*.



He proposed the following structural explanation of the sources of the different products.

(7a)



According to this explanation of Harden's, the center groups  $\text{CHOH} \cdot \text{CHOH} \cdot \text{CHOH}$ , which have the same empirical formula as lactic acid, yield this substance by the interchange of a hydrogen and an

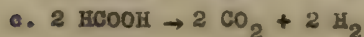
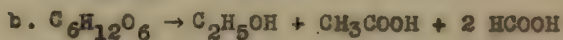
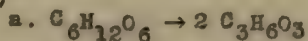


oxygen atom between the two terminal groups, or between the terminal groups of a second similar chain derived from another molecule of the hexose.

This equation, however, is not meant to represent accurately the fermentation of even these hexoses whenever they undergo "acid gas lactic acid fermentation". Harden and Walpole (1906) found that the proportional quantities of the products varied not only with closely related species but even with strains of the same species. He explained this from the basis of equation (7a), as the result of secondary changes, in which part of the three center CHOH groups break down into alcohol, acetic acid, succinic acid, hydrogen, and carbon dioxide. The variation is, however, more easily explained by a later proposal of this authority.

In 1912, Harden and Penfold stated that the reactions involved in these mixed lactic acid fermentations could be best represented by the following equations, in which the different products arise from more or less distinctly separate reactions.

(8)



By this theory, the variations in products may be explained upon the basis that these different reactions operate more or less independently in the same cell. In different species these reactions may possess different velocities; with the same organism, by varying the conditions, such as that of the character of the other nutriments, one or the other of these simultaneous reactions may be favored. In this way a varying proportion of the products would be formed in different systems.

Recent work seems to establish Harden and Penfold's contention that more than one reaction is involved in acid gas lactic acid fermentation. Kamm's work strongly suggests at least two simultaneous, but independent, reactions. Grey, (1918), believes that there are two main reactions. His recent investigation shows that the "fermentation takes place in two main directions. On the one hand, formation of lactic acid and on the other hand, formation of a group of substances (alcohol, acetic acid, gas), which seem to be related more closely to one another as regards their origin, than they are related to lactic acid."

In 1920, Grey gives still further and quite conclusive evidence of the independence of these reactions, especially the production of lac-

tic acid. He shows that the "mixed fermentation" yields products which seem to be divided into three main groups: (1) Lactic acid; (2) Acetic acid, alcohol, and succinic acid; (3) Carbon dioxide, hydrogen, and formic acid. There seems to be a closer relation between the second and third groups than between either of these and the first group. The availability of hydrogen seems to determine the proportionate production of alcohol, succinic acid, and acetic acid (in the order given).

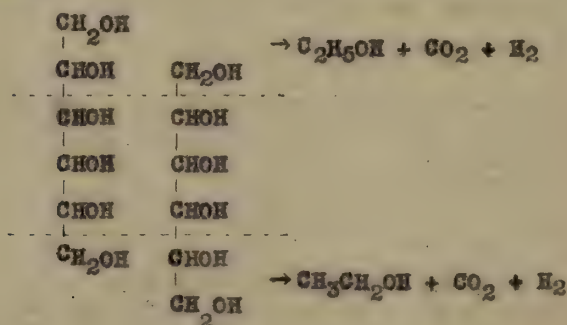
b. Other "mixed" lactic acid fermentations.  
of hexoses.

The above work of Harden and associates, and of Grey have been confined to the most common type of "mixed" lactic acid fermentations of hexoses. These compounds, however, are attacked by certain other lactic acid bacteria, by means of other types of reactions. Moreover, the recent work of Fred and associates confirms earlier suggestions that there may be different chemical changes involved in the fermentation of the different hexoses. This has not been evident in the group of acid gas fermentations discussed above, but, with certain other lactic acid bacteria, the fermentation of fructose at least seems to follow a different course of reactions. (See also "Enzymes"). Further material on the mixed fermentations of different hexoses given

by certain pentose destroying bacteria, with a discussion of the chemical changes and reactions involved, may be found in the reports of Fred and associates.

c. Acid gas fermentation of mannitol.

Besides the hexoses and disaccharoses, other carbohydrates undergo lactic acid fermentation. Their fermentation probably is not so important in agriculture, but below is given the structural equation proposed by Harden (1901) for the fermentation of mannitol.



In this case, the two center "residual groups" suffer greater secondary change than in equation (7), and correspondingly less lactic acid is formed and more of other products.\*

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\* See "Other Products".



#### d. Fermentation of pentoses.

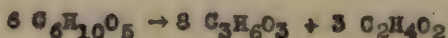
The fermentation of pentoses is included in this paper under the "mixed" lactic acid fermentations, although gases are not so frequent a product as in the group of fermentations discussed above.

However, other acids than lactic acid, chiefly acetic, are produced in considerable amounts.

Jensen found several groups of his lactic bacteria fermented pentoses. He reports (1919) other substances, usually acetic acid, are constantly produced along with lactic acid. He points out that it is impossible for a five-carbon sugar to yield two molecules of lactic acid by an intramolecular reaction, as do the hexoses. In the products of their fermentation, he found more lactic acid and less acetic acid than would answer to the equation.



He found, however, that some of his lactic acid bacteria fermented arabinose with products corresponding to the following equation:

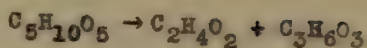


Arabinose

The fact that the relative amounts of acetic and lactic acid vary with different conditions, even with the same fermenting agent, strongly suggests that here, too, we have to deal with more or less simultaneous reactions, as proposed above by Harden and Penfold and Grey, in case of fermentations of the acid gas type.

Fred and associates (1919 and 1920) have recently studied the fermentation of pentoses with special reference to production of acetic acid. They found that the chief products arising from fermentation of xylose are lactic and acetic acids, 90% to 95% of the xylose consumed being accounted for by these products. The ratio of acetic to lactic acid approaches very near the theoretical figure, from

the equation below:--



$$\frac{\text{lactic acid}}{\text{acetic acid}} = \frac{90}{60}$$

A more complete discussion, together with references to the literature, of pentose fermentation may be found in the reports of Fred and co-workers.

No very satisfactory structural explanations have been presented for the lactic acid fermentations of pentoses, although Harden (1901) suggested a structural equation for this reaction somewhat similar to the one given above for the six carbon sugars.

#### 4. Role of intermediate substances.

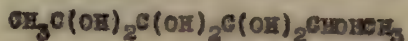
##### a. Introduction.

Although the above empirical equations show merely a direct conversion of dextrose to lactic acid, there is a decided tendency in recent years to believe that other transient products are first formed from the sugar, and that these intermediate substances are then converted into lactic acid. Both the formation of these substances and the subsequent formation of lactic acid involve the temporary entrance of  $\text{H}_2\text{O}$  molecules and the migration of hydrogen and oxygen atoms, which were evident in the above discussions.

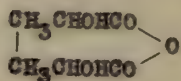
b. Bayer's anhydride theory.

Some authorities support Bayer's, (1870), early explanation of the formation of lactic acid as an intermediate substance in alcoholic fermentation.

An accumulation of oxygen atoms takes place in the center carbon groups of the dextrose molecule, which is then changed to the form below:--



This form loses two molecules of water, and, by a migration of a hydrogen atom from the hydroxyl group left between the two middle carbon groups, lactic anhydride is formed.



The anhydride, by taking up a water molecule, is changed to two molecules of lactic acid.

c. Trioses as probable intermediate substances.

In later investigations, trioses appear to be more probable intermediate substances.

Wohl, (1907), proposed three carbon compounds as intermediary substances in alcoholic fermentation. He believed that glycerin, aldehyde, and methyl-glyoxal were formed during the change of dextrose to lactic acid. Nef, (1904), has proven the formation of methyl-glyoxal (pyruvic aldehyde) as an intermediate substance in the alkali splitting of dextrose to lactic acid, and believed it probable that the same substance is involved as an intermediate product in lactic acid fermentation. Buchner and Meisenheimer, (1905), although in an earlier paper, (1903), they had proposed the formation of a hypothetical diketonic acid, were inclined to agree with him. Nef (1904) also observed formation of glycerin aldehyde and carried out experiments proving the formation

of lactic acid from that substance by action of alkalis. Dioxycetone, which was proposed by Buchner and Meisenheimer (1910) as an intermediate substance produced between dextrose and lactic acid in alcoholic fermentation, is also considered a probable intermediate substance in lactic acid fermentation.

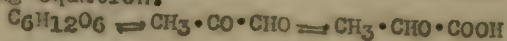
These three substances, --- methyl-glyoxal, glycerin aldehyde, and dioxycetone --- are, from a theoretical standpoint, quite readily formed from glucose. (For a more complete discussion of this point, see the references given, also Beatty (1917), Harden (1911), and Euler and Lindner (1915).). From a consideration of their structural formulae, it is evident that the formation of lactic acid is possible by hydration, (Beatty), or by a very simple migration of hydrogen and oxygen atoms within these molecules, (Embsen, Balden and Schmitz).

The following investigations further support the probability of the entrance of these three-carbon-group compounds as intermediate substances in the change of lactic acid to glucose.

M. Oppenheimer (1912) has shown that these proposed intermediate substances are changed into lactic acid by action of sodium hydroxide, as employed in the alkali splitting of dextrose, and that the production of lactic acid from certain of these substances is much more rapid than that resulting from similar action on dextrose. Embsen, Balden and Schmitz (1912) proved the formation of lactic acid in the animal body from glycerin aldehyde and dioxycetone. Levine and Meyer (1913) have demonstrated the conversion of methyl-glyoxal (pyruvic aldehyde) to lactic acid by action of leucocytes and kidney tissue. Dakin and Dudley's (1913) work indicates that this substance is an intermediate stage between glucose and lactic acid, when the change occurs in the animal body. They believe that



the conversion of methyl glyoxal into lactic acid is a reversible reaction, and represent the relationship between glucose, methyl glyoxal and lactic acid, by the following equation:



Neuberg and Oertel (1913) demonstrated the production of methyl glyoxal from sugars of the six Carbon series by action of weak alkalis; Neuberg and Rewald (1915) showed that the production of methyl glyoxal is not limited to action upon hexoses, but that it is also yielded by similar treatment of pentoses, disaccharides and other carbohydrates. Neuberg believes that reactions similar to the Cannizzars reaction are involved in lactic acid fermentation, and that methyl glyoxal serves as an intermediate stage in the transformation of glucose to lactic acid. (Neuberg and Kerb (1915)).

From the weight of evidence presented, Bayliss (1915) concludes that glyceric and pyruvic aldehydes (methylglyoxal) are intermediate stages when glucose is changed to lactic acid in the animal body.

Dakin (1921) believes that either of the above two substances or more probably optically active hydrates of methylglyoxal, are intermediate stages in the conversion of carbohydrates to lactic acid.

Whether the same order of reactions taken place in the microbial production of lactic acid is impossible to state.<sup>#</sup> However, Herzog and Horth, Czapek and others, believe that results of investigations indicate that three-carbon compounds, such as

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<sup>#</sup> Many investigations have been made on the rôle of trioses, as intermediate substances, in the alcohol fermentation of hexoses. (See Marden, 1911, and Euler and Lindner). These have yielded much of value to the interpretation of that fermentation. The rôle of similar substances in the production of lactic acid by animal tissues has also been investigated with positive results. It seems that an investigation of the same intermediate substances, from the standpoint of microbial lactic acid fermentation, should yield contributions of importance, not only to a knowledge of the mechanism of the fermentation process itself, but also to a correct interpretation of many economically important agricultural processes.

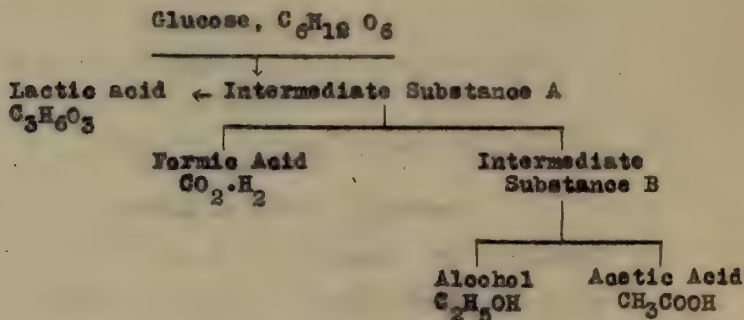
the above substances, are involved as intermediate steps in the formation of lactic acid from dextrose in lactic acid fermentation. Some authorities, (Thom and Fisk, and others), suggest this as a possible explanation of the increase of the lactic acid content of ripening Cheddar cheese, after the exhaustion of lactose.

In his discussion of the probability of lactic acid serving as an intermediate substance in alcoholic fermentation, Cohen (1910) presents a statement that is of equal pertinence to the question of the "intermediate" role of three Carbon compounds in lactic acid fermentation. He shows that there is always a possibility of the "potential" formation of intermediate substances in the rearrangements of a sugar molecule, but that these intermediate substances may be recognized only if the reaction be stopped at that stage. It is also true, of course, that intermediate substances would not be detected if the reactions attacking them proceed at greater velocities than those responsible for their production.

Reports of investigations on the question of lactic acid as an intermediate substance in alcoholic fermentation are given later in this paper. (See "Enzymes") These (many of which appeared after Cohen's statement) emphasize the significance of his remark.

d. Intermediate substances in acid gas lactic acid fermentation.

Gray (1914) proposed the formation of intermediate products in lactic acid fermentation of glucose by *B. coli*. He represents them as follows:-



In the case of mannitol, he suggests a similar process, with the exception that the two hydrogen atoms possessed by the mannitol in excess of those of the glucose molecule, are set free at the time Intermediate Substance A is produced. Although Intermediate Substance A is unknown, Gray believes it to be closely related to pyruvic aldehyde (methyl-glyoxal). The essential difference in the acid gas fermentation, of these two carbohydrates, he explains by the reducing action of these two hydrogen atoms, which reaction gives rise to a production of alcohol\* in excess of that produced from glucose.

In later work (1920), he actually demonstrated that nascent hydrogen does take part in the reaction, particularly in the production of alcohol. He showed that the same is true, not only in the fermentation of mannitol, but also in that of glucose. By adding calcium formate to glucose fermentation mixtures, he showed that nascent hydrogen is an active agent in the fermentation, not only when the hydrogen arises from the decomposition of the glucose itself, but also when hydrogen is supplied in the nascent condition by the simultaneous fermentation of formic acid added to the system.

\* See "Other Products" --- Alcohol.

Petersen and Fred (1920), and Fred and associates (1920) believe that malic acid may sometimes serve as an intermediate substance in the production of lactic acid from sugars in mixed lactic acid fermentations. They suggest the following equation to represent the changes taking place:--



## II. Energy Changes Involved in Lactic Acid Fermentation.

### 1. Thermodynamics of lactic acid fermentation.

#### a. Source of energy required.

In the first chapter, it was established that lactic acid fermentation is produced by the life processes of micro-organisms. Therefore, in order that the catalytic reaction be induced, it is necessary that the microbial agents be provided with energy, not only for growth and multiplication, but especially for the carrying on of those life processes by which they elaborate the catalyst of the reaction.

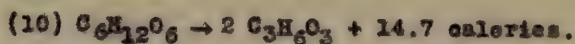
"The source of energy in microbial life is always of chemical origin." (Rahn). The fermentation of sugars is the dominant chemical change produced by lactic micro-organisms, and it is known that the chemical change involved in lactic acid fermentation of sugars takes place within the cell. (See "Enzymes"). Hence, it may well be



considered that this reaction is the principal source of energy of the microbial agents of lactic acid fermentation. The thermo-chemical equations of these changes, then, should represent the energy provided by the lactic acid fermentation reaction, for the growth, reproduction, and other life processes of lactic micro-organisms.\*

b. Energy yielded in lactic acid fermentation.

Heraeg (1903) gives the following energy equation for the "true" lactic acid fermentation of dextrose.

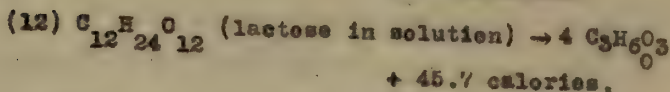
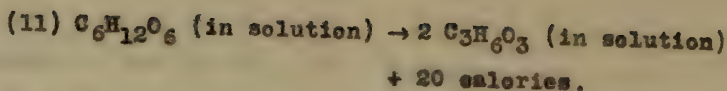


Heat of combustion	(Dextrose	673.7 cal.
	(Lactic acid	659.0 cal.
	$329.5 \times 2$	
		<hr/> 14.7 cal.

Berthelot obtains slightly higher results from calculations upon the basis of the dextrose and lactic acid being in solution:--

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\* There are other sources of energy in the system, but the reaction of lactic acid fermentation is essentially an endocellular process and probably furnishes practically all of the energy used by the lactics. Heat of neutralization, et cetera, probably is valueless to the micro-organisms. In his thermometric investigation of spontaneous lactic acid fermentation of milk, Rubner (1906) observed a greater thermal effect than could be explained by the lactic acid fermentation of lactose. This was not a pure culture and being kept at 37° C., the fermentation was probably largely due to the acid gas group, which reaction yields more energy.



1363.7 cal.

1318.0 cal.

45.7 cal.

By comparing these equations with those of processes of complete oxidation, it is evident that much less energy is yielded by the lactic acid fermentation of a sugar molecule. Hence, the micro-organisms of lactic acid fermentation require much more food than do micro-organisms taking part in more productive reactions.\* The figures below show the energy liberated when one gram of dextrose is subjected to the following biochemical reactions.

1 gm. dextrose

Complete oxidation ( <i>Cidium lactis</i> )	3750 cal.
Butyl alcohol fermentation	
( <i>Granulobacter butylic</i> )	
( <i>B. orthobutylicus</i> )	210 cal.
Alcoholic fermentation (Yeasts)	122 cal.
Lactic acid fermentation	
("True" lactic acid bacteria)	80 cal.

(Herzog's figures).

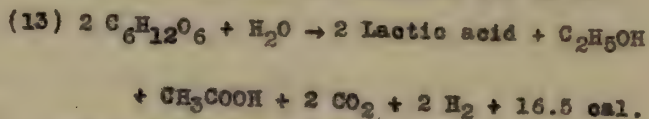
These figures show that a much larger amount of sugar must undergo the reaction of lactic acid fermentation than that which would produce the same amount

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\* Rahn calculated the amount of sugar fermented per hour by common lactic acid bacteria to be about equal to the weight of the cell.

of energy in one of the other reactions.

In lactic acid fermentations in which lactic acid is not the only product formed in significant amounts, these equations would not apply.\* Kruse has calculated the energy yield of the acid gas fermentation reaction of equation (7) to be



This thermochemical equation is merely the algebraic sum of the thermochemical equations of the several fermentations of equation (8). Naturally, therefore, the energy yield would depend upon the relative predominance of the reactions involved in these mixed fermentations.

## 2. Chemical nature of the fermentation process.

### a. Rearrangement of matter and of energy involved in the reaction.

The chemical change of fermentations, such as lactic acid fermentation, is often termed molecular rearrangement. "Every chemical change consists in simultaneous rearrangements of matter and of energy. The true nature of the chemical process is to be sought neither in the one nor in the other of these two phenomena, but in both together." (Henderson). That there has been a rearrangement

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\* As for instance, in Rubner's spontaneous fermentation of milk at 37° C..

of matter in lactic acid fermentation is evident from a comparison of the structural formulae of glucose and lactic acid; it is with the rearrangement of energy that the following discussion is primarily concerned.

In the preceding discussion of the chemical changes involved in lactic acid fermentation, it was intimated that, although oxygen does not enter into the reaction formula, there seem to occur certain reactions of oxygen atoms within the hexose molecule. Notwithstanding the fact that the empirical formula of the six carbon group glucose molecule contains the same proportion of hydrogen and oxygen atoms as do the three carbon group lactic acid molecules, their structural formulae show that the end carbon groups of the lactic acid and glucose molecules differ in their oxygen and hydrogen content. In each molecule of the lactic acid, one of the end carbon groups has been oxidized to a carboxyl group at the expense of the other, which has been reduced to a methyl group. This rearrangement of matter results in the formation of a compound containing less potential energy than did the sugar molecule; there has been in this way a rearrangement of energy. The excess energy is transformed into kinetic energy, which is available to the use of the micro-organisms.



b. Comparison of oxidation and fermentation.

The complete oxidation of a glucose molecule yields 673.7 calories,



while that of lactic acid fermentation yields but 14.7 calories. The very great difference in the rearrangement of energy involved in the two reactions may be explained by the difference in the rearrangement of matter which takes place in each case.

(1) "Intramolecular oxidation".

Fermentations are sometimes termed "intramolecular oxidation" as a convenient means of expressing the migration of oxygen and hydrogen atoms which takes place between the carbon groups. The above paragraph stated that one carbon group had been oxidized at the expense of another carbon group of the hexose molecule. It is this fact that explains the first fundamental difference between fermentations and oxidations.

In the complete oxidation of glucose, the necessary oxygen is furnished from the surroundings of the molecule. In lactic acid fermentation, the oxygen which is required for the oxidation of one carbon group to the carboxyl form is furnished from within the molecule, and a large part of the energy yield, which would result from the oxidation of one carbon group, is absorbed by the consequent

reduction of another carbon group to the methyl form. It is only because the energy liberated by the rearrangement of matter to form the COOH group exceeds that absorbed in the formation of the CH<sub>3</sub> group that the reaction of lactic acid fermentation is an endothermic reaction at all.

(2) Degree of completeness of the energy transformation.

The second and perhaps more important difference is found in the energy content of the reaction products, or the degree of completion of the transformation of potential into kinetic energy. In lactic acid fermentation the rearrangement of the energy of the matter in a hexose molecule results in the formation of a compound possessing a combustion heat of 659 calories. In complete oxidation of glucose, the reaction products possess no combustion heat, and all of the potential energy of the hexose molecule is set free. Thus, the energy transformation in lactic acid fermentation is very much less than in complete oxidations.

The high energy content of lactic acid itself makes lactic acid fermentation a reaction of very incomplete energy transformation, even when compared to other fermentation processes. In alcoholic fermentation, no oxygen is furnished from the surroundings, but the reaction products formed include carbon dioxide, a product of complete ener-

gy transformation.\* This is the cause of the greater energy yield of this fermentation.

Further comparison with other fermentation processes, showing still more complete energy transformations, makes even more evident the incomplete degree of energy transformation attained in the lactic acid fermentation reaction. However, the energy still bound up in the lactic acid molecules is not lost to the system, as will be shown later.

### 3. Biological nature of the fermentation process.

#### a. Physiological significance of the energy change of the lactic acid fermentation reaction.

Lactic acid fermentation is a biochemical reaction; it involves rearrangement of both energy and matter. The chemical changes of perhaps most life processes of organisms are concerned with transforming food into a form more readily utilized by the cell (e.g., most hydrolyses). Many of these changes are concerned entirely with the rearrangement of the matter contained in the food molecules; in these reactions the energy changes are incidental. Exactly the opposite relation is shown in the relation of the lactic acid fermentation reaction to the life processes of the cell.

\* The transformation of lactic acid into alcohol and carbon dioxide is itself a reaction which yields a little heat (8 C at 30°, according to Mazé's citation of Barthelot's results). From thermochemical laws, this fact in itself would require a greater yield of energy in the production of alcohol and carbon dioxide from a molecule of glucose than would be liberated in the production of two molecules of lactic acid.

In lactic acid fermentation, the physiologically significant change is the rearrangement of energy. The energy transformation of the exothermic reaction is fundamental to the life of the lactics, while the rearrangement of matter, which results in the formation of molecules of a substance of little, if any, food value, is more or less incidental (Oppenheimer, 1913). Then, in the reaction of lactic acid fermentation, the essential change is the energy transformation; its essential rôle is the furnishing of kinetic energy to the micro-organisms which elaborate the catalyst of the reaction.

#### b. Relation of oxygen to life.

Pasteur's first definition of fermentation, "Fermentation is life without oxygen", is perhaps a too inclusive statement. Reactions of oxygen atoms are such productive sources of energy that it is by this means that most micro-organisms secure the energy they require. In lactic acid fermentation, it may be assumed that fermentation of a sugar involves reactions of oxygen atoms within the molecule. If no oxygen from the air is available, the fermentation of the sugar, (with its probable intramolecular oxidation reactions), is necessitated, in order that energy be furnished the organism. The dependence of all organisms upon either



fermentation or oxidation reactions for sources of energy is shown by the fact that it has never been proven that any micro-organism can maintain life in the complete absence of both free oxygen and a fermentable substance.\* Even in the case of yeasts, the micro-organisms, to which Pasteur applied the above definition, this is found true. From this standpoint, Oppenheimer (1913) would restate Pasteur's definition as follows: Fermentation is "essentially a means of obtaining the energy required for life in the absence of oxygen"; Kruse, "Life without oxygen is possible only through fermentation."

#### c. Comparison of fermentation and respiration.

From an energetic standpoint, respiration and fermentation bear the same relation to each other as that shown above, between oxidation and fermentation. Examination of the first named processes shows that here, too, the difference is largely one of degree. From the biological standpoint, fermentation is often termed "intramolecular respiration", just as from the chemical standpoint it is sometimes

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\* This need not always be a carbohydrate, although that is the usual substrate.

called "intramolecular oxidation".\*\* A complete discussion is out of place in this paper, but the following quotation from Jost shows the close analogy between these relations.

"The destructive metabolism in so far as it consists in the complete combustion of organic materials with production of carbon dioxide and water, is termed respiration. By fermentation we mean an active metabolism where the oxidation is incomplete, or where, instead of oxidation, a decomposition of a different kind takes place.

"Respiration and fermentation have in common the formation of final products having less heat of combustion and more limited stores of energy than the materials from which they arise. In the formation of these final products, energy must, therefore, be released, and it is this energy which the organism uses to carry out its vital activities.

"Respiration and fermentation are not two essentially different processes, for, since in the respiration of fungi, for example, a number of organic acids arise as products of incomplete combustion, we are quite entitled to term this process a fermentation."

#### d. Biological significance of the degree of the energy transformation.

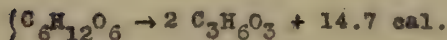
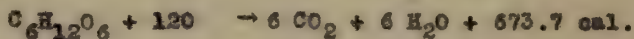
The fact that the energy content of the system (i.e., that part of it furnished by the sugar) is incompletely transformed is of biological signifi-

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\*\* The assumption of some plant physiologists that carbon dioxide is always a product of "intramolecular respiration" is unfounded, as is proven in the case of lactic acid fermentation. The difference between this fermentation and the fermentations in which carbon dioxide arises is merely a difference in degree of energy transformation, or a difference in degree of oxidation, just as that separating the so-called "fermentations" from "oxidation processes." (Jost; compare Kruse).

cance. Among other cases, this is evident in mixed cultures in which molds and lactic acid bacteria are present in "serial association". That part of the initial energy of the sugar which remains in the lactic acid molecules is still available to molds such as *Oidium lactis*.

In conformity with the law of Hess, the complete oxidation of the product of lactic acid fermentation will yield the energy difference between that of the complete oxidation of glucose and that transformed in lactic acid fermentation.



The last equation shows that there is a very productive source of energy still available in systems in which lactic acid transformation has taken place. The degree of energy transformation is probably likewise significant in all intermicrobial relations.

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C. THE LACTIC ACID BACTERIA.

## THE LACTIC ACID BACTERIA

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a. History.

b. Identity and nomenclature.

3. Salient characters of the Group.

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V. *Lactobacillus* Group.

1. Micro-organisms included.

2. Type organism.

3. Sub-groups and distinctions within the Group.

a. Types recognized.

b. Systems proposed.

4. Salient characters of the group.

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5. Distribution and source.

VI. Fourth Group of Lactic Acid Bacteria.

1. Micro-organisms included.

2. Salient characters.

a. Morphology.

b. Physiology.

3. Distribution and source.

VII. Other Lactic Acid Producing Bacteria.

VIII. Lactic Acid Production by Other Organisms.

## THE LACTIC ACID BACTERIA

### Introduction.

The results of the investigations reported in the first chapter established the fact that lactic acid fermentation may be brought about by certain micro-organisms. In the investigations there reported, several different micro-organisms had been isolated, which were capable of inducing lactic acid fermentation, (that is, of acting as biochemical catalysts in the chemical reactions just discussed). The following chapter will have to do with a further study of the micro-organisms which are the active agents of the fermentation process.

### I. Definition of Lactic Acid Bacteria.

The same difficulties that are encountered in a definition of lactic acid fermentation prohibit a strict definition of lactic acid bacteria. The biological agents of lactic acid fermentations must be included in such a definition. It should not, however, include all micro-organisms which produce lactic acid. This latter treatment would introduce many organisms in whose sum total of activities the lactic acid fermentation reaction is not a significant life process. An exact demarcation between the groups obtained by these two treatments is impossible to make.

As a result, different authorities vary in their conception of the meaning of the term "lactic acid bacteria". Some would include all bacteria which are able to produce lactic acid from any carbohydrates, others, only those which attack lactose with production of lactic acid. Others differ in their conception of

the micro-organisms included in this category, largely upon the basis of the particular type of fermentations investigated. Lactic acid fermentation is so intimately associated with the dairy industry that most of the investigations of the process have been made in this field. This fact probably accounts for the common tendency to include among lactic acid bacteria only those common in the fermentation of milk. Further research in the fermentations of plant products and in the microbiology of soil will probably demonstrate the fallacy of this conception. Prolonged discussion of the proper boundaries of the term "lactic acid bacteria" would be unproductive. Nothing more will be attempted than a statement of the limits placed by the writer in his treatment of the present paper.

Lactic acid bacteria, in this paper, include those micro-organisms which derive a significant and usually predominant portion of their energy from the reaction of lactic acid fermentation, when in systems presenting a carbohydrate fermentable by their enzymes. Whether this category will include micro-organisms of close phylogenetic relations is impossible to state. It seems, however, that basing the determinant character of the group upon the source and means of obtaining energy, (their most fundamental requirement), should furnish a fairly natural group of

micro-organisms. It is certain that even agricultural lactic acid fermentation cannot be determined by the boundaries of the dairy industry --- no more so, the active agents of this process.

This treatment will give a more inclusive and also more unwieldy group of bacteria than a group limited to a special applied field of lactic acid fermentation. This will be evident in the following discussion of the methods of dividing the large group of lactic acid bacteria.

## II. Grouping of the Lactic Acid Bacteria.

### 1. Difficulties encountered.

The study of the large number of species and strains of micro-organisms that may be considered as lactic acid bacteria is most conveniently approached by placing them in more or less indefinite groups. Any such grouping of living beings is difficult and more or less arbitrary. This usual difficulty is increased by the large number of species of lactic acid bacteria and of strains or types within the same species; by the gradations existing between the members of the different groups and <sup>by</sup> the slight distinctions possible to make between many members within the different groups. These difficulties are again multiplied by the indefinite descriptions and confused terminology applied to these bacteria by the earlier investigators. The different



systems proposed in the literature will be given below.

## 2. Systems proposed.

McDonnell (1899) and Weigmann (1899) proposed a classification of lactic acid bacteria based on their action in milk. Their studies include only those lactic acid bacteria encountered in the dairy.

Conn, Esten and Stocking, in their classification, (which, strictly speaking, is not a classification of lactic acid bacteria, but rather, of milk bacteria), based their groupings and differentiations upon the generic relations of the organisms, such as possession of flagella and the usual cultural characteristics of the systematic bacteriologist.

Gorini has grouped the lactic acid bacteria according to physiological characters, such as gas production, temperature relations, acidifying power and proteolytic action.

Upon a somewhat similar basis Rogers and Davis divided the lactic acid bacteria of milk into the following four classes:

- I. Sour milk, without peptonization and gas formation.
- II. Acid curd, with gas.
- III. Acid curd, subsequently peptonized.
- IV. High acid formation; long rods.

The organisms within these groups were further subdivided by other physiological properties; among these,

they considered action on raffinose, glycerin or gelatin most valuable for differentiation.

Their four large groups offer the most convenient method of treatment of the lactic acid bacteria of milk.

The grouping given below is that suggested by Löhnis (1907, 1910).

- I. *B. aerogenes* (*B. acidilactici* Hueppe) group.
- II. *Strep. lacticus* (*B. lactis acidilichmanni*) group.
- III. *B. bulgaricus* or *Bact. caucasicum* group.
- IV. Micrococci group.

It will be noted that Löhnis' large groups are based upon much the same characters as those used by Rogers and Davis. His first group corresponds to the second group of the Americans' classification. His second and third groups produce the "true lactic acid fermentation" of Duclaux and Kayser, as do Rogers and Davis' first and fourth groups. Löhnis then further subdivides these groups upon such characters as slime production, proteolysis, etc..

Although accepted, at least in part, by most authorities, Löhnis' system is perhaps almost too inclusive for a convenient working basis. Leichmann (1907) criticized it in that respect, pointing out that the second and fourth groups might as well include all existing micro- and strepto-cocci.

The difficulties to be encountered in establishing a comprehensive but workable grouping of lactic acid bacteria are evident in the reception of Löhnis' system. It has been criticized chiefly for its wide boundaries, yet even his system does not recognize all the types important in certain lactic acid fermentations.

Löhnis' fourth group is exceedingly difficult to handle in a systematic manner, and has consequently been conveniently ignored by many authorities. By eliminating this as a group and by making the first three groups adhere more closely to their respective type species, a system of lactic acid bacteria is obtained which includes the majority, but not all, of the important agents of agricultural lactic acid fermentation. Kayser advances such a system, which, in some ways, is preferable to the more inclusive system of Löhnis. Such treatment, however, is also open to criticism and is hardly suited for a working basis for the presentation of this paper.

Beierjink and Jensen (1914, 1915) have set up groupings of lactic acid bacteria based upon their temperature relations. Jensen's system is given below.

- I. Lactic acid bacteria growing only between 25° C. and 50° C.  
All long rods and almost always form levo acid.
- II. Lactic acid bacteria growing as well at 5° C. to 7° C. as at higher temperatures (45° C. to 50° C.).

These are all streptococci (e.g., the *Mazun strep.*).

- III. Lactic acid bacteria growing only at medium temperatures (seldom below 10° C. or above 40° C.).  
Most of the true lactic acid bacteria belong to this class.

Such a system can at best serve only the most general needs.

Recently, Jensen (1919) has proposed a more comprehensive system, based on his valuable study of the metabolism of lactic acid bacteria.

A. Without catalase, reduction of nitrate and surface growth:

- a. Produce only a trace of by-products besides the lactic acid.

Genus: *Streptococcus* --- always form dextro lactic acid and thrive well in milk, but not as well, or even badly, in yeast extract.

- α. Mostly shorter or longer chains.  
Never pentose fermentation.

Species: *thermophilus*  
*cremoris*  
*mastitidis*  
*pyogenes*

- β. Diplococci as well as longer chains.  
Mostly pentose fermentation. Always ferment maltose, dextrin and salicin, as a rule also saccharose.  
Maximum temperature, 45° C.

Species: *liquefaciens*  
*glycerinaceus*  
*inulinaceus*  
*bovis*

- γ. Mostly diplococci. Always ferment maltose, dextrin and salicin; mostly pentoses, also.

Species: *faecium*  
*lactis*



Genus: *Thermobacterium*. Form levo or inactive lactic acid. Except *Thm. cereale*, they strongly break down casein and thrive well in yeast extract. They never ferment pentoses and frequently, not salicin. Long rods, which grow at 50° C. or more, but do not, on the other hand, grow at temperatures lower than 22° C.

Species: *helveticum*  
*Jugurt*  
*bulgaricum*  
*lactis*  
*cereale*

Genus: *Streptobacterium*. Form inactive or dextro lactic acid. Thrive well in yeast extract and as a rule also in milk. Always ferment maltose and salicin and usually lactose. Short or long chains. Maximum temperature, usually 35° C. to 40° C.

- b. As a rule, produce perceivable amounts of gas and other byproducts besides the lactic acid.

Genus: *Betacoccus*. Form levo lactic acid, or exceptionally, inactive lactic acid. Mostly form slime from saccharose. Thrive well in yeast extract, but only now and then well in milk. Never ferment inulin and starch, rarely dextrin. Frequently ferment raffinose. Low optimum temperature.

Species: *arabinosaceus*  
*bovis*

Genus: *Betabacterium*. Almost always form inactive lactic acid. Thrive well in yeast extract, but as a rule, badly in milk. Never ferment considerable amounts of mannite, inulin, dextrin, starch, or salicin. Comparatively small mannose fermentation.

Species: *caucasicum*  
*breve*  
*longum*

B. As a rule, with catalase, reduction of nitrate, and surface growth.

Genus: Tetracoccus. Dextro lactic acid.  
Division in two or three planes.

Species: casei  
liquefaciens  
mycoderma

Genus: Microbacterium. Dextro lactic acid.  
"Thrive badly in yeast extract."  
Very small rods, barely more than  
0.5 microns thick.

Species: lacticum  
mesentericum  
flavum

Jensen's choice of characters for differentiation, and choice of terms to represent the species so obtained are certainly not above question. His classification of streptococci is particularly audacious. It is certain that he has not added to the value of his worthy study of the biochemical activities of lactic acid bacteria by assigning these to illy-defined species, rather than to type groups.

Winslow admits the value of Jensen's work, but criticizes it justly from a systematic standpoint. "The evidence for combining the streptococci and the Bulgarian bacillus in one family is suggestive, but hardly conclusive; while, as in previous communications, Jensen appears entirely innocent of any knowledge of the principles of biological nomenclature or of any respect for the work of previous investigators. His genus Tetracoccus is apparently Lereconostoc of Van Tieghem and his Thermobacterium is certainly Lactobacillus Beijerinck; while many of his specific names are merely confusing synonyms of perfectly valid names given by previous investigators."

Heinemann (1920) also comments on the doubtful systematic fitness of Jensen's system.

In addition to its evident disregard of established nomenclature, Jensen's system of grouping the lactic acid bacteria does not furnish a convenient basis for the treatment of this paper.

A study of lactic acid fermentation cannot readily omit the acid gas fermentations and consequently it seems that a system of lactic acid bacteria should

include the acid gas group. Work shown already, under "Chemical Changes", indicates that the lactic acid fermentation by this group is not essentially different from that of the so-called "true lactic acid bacteria". Apparently, the lactic acid fermentation reaction is brought about by this group much in the same way as in "true" lactic acid fermentation, with the exception that other independent reactions also accompany it. Moreover, the grouping of lactic acid bacteria should be merely a convenient means of studying lactic acid fermentation. From this standpoint it certainly does not seem logical to exclude a group which are so important in practically all aspects of lactic acid fermentation as are Jensen's "pseudo lactic bacteria".

3. Inadvisability of attempting a systematic classification of lactic acid bacteria, as such.

It is unwise and apparently impossible to set up a systematic classification of lactic acid bacteria. It seems that there is little to choose between a comprehensive, but unwieldy, system and a simpler, but non-inclusive, system.

Perhaps all that should be attempted is a grouping of the fermentations themselves, based upon the products of the reactions upon the substrate. The active agents of these processes should then be described in the standard terminology of the workers in the other fields of microbiology.

4. Groupings of value only as a working basis.

It is certain that any grouping proposed for the large number of types of lactic acid bacteria will be cumbersome, if it includes the agents of all aspects of this fermentation. Any grouping of these organisms is of value chiefly in the study of lactic acid fermentation. At best it can only set up certain groups as including the micro-organisms responsible for different types of fermentations obtained in certain fields. The lactic acid bacteria of plant products would probably not fall into the groupings found convenient in a study of the fermentations of milk and milk products.

As a working basis, Löhnis' system is usually used. For the worker in the dairy aspect of lactic acid fermentation, it is probably not as serviceable as the simpler one of Rogers and Davis. In this paper, Löhnis' system is chosen, merely because it is in general use. Other groupings would be found more convenient in studies of special fields of lactic acid fermentation.

No attempt is made here to describe extensively any of the lactic acid bacteria in regard to morphology or cultural characters. In the following consideration of the groups of lactic acid bacteria, nothing more is attempted than a statement of the salient features of each group, and its most important members. Reference to the literature cited in the text will furnish an extensive treatment.



### III. Acid Gas Group.

#### 1. Micro-organisms included.

This group is also sometimes called the *Aerobacter* Group (Beierjinnk), or, from name of organism usually considered as the type species, the *B. aerogenes* (Kruse) or *Bact. acidilactici* (Hueppe) group. It includes a large number of species and strains. Among the most common members are the type species, *B. aerogenes* or *Bact. acidilactici*, and its close relative, *B. coli*.

#### 2. Type species.

The use of the term "type species" is more or less unsatisfactory, as it is intended to represent merely a type of the members of the group which are most common in agricultural lactic acid fermentation. There is little reason for choosing this type of acid gas bacteria in place of the *B. coli* type, other than its apparently greater ubiquity in agricultural products.

##### a. History.

*Bact. acidilactici*, Hueppe, (*Bact. (lactis) aerogenes*, Escherich, *Bact. aerogenes*), is taken as the type species of this group. This organism was the first lactic acid bacterium to be isolated by the Koch method. For this reason, and because of the claims of its isolator, it was assumed for some time to be the true <sup>type</sup> species of the organisms causing lactic acid fermentation. This assumption was unfortunate, since the acceptance of the fermentation induced by this organism as the typical lactic acid fermentation led to the wrong conception of that process. Later, the type species of our second

group was isolated and given a name closely resembling that used by Hueppe for his bacillus (Leichmann). From these unhappy choices in names arose much of the great confusion still existing in the nomenclature and systematics of the lactic acid bacteria.

Much of the early literature is concerned with disputes over the identity and nomenclature of the type species of this group and with the controversy over the relation of this organism to the type species of the following group. A review of these points, together with discussions of the rôle of these lactic types in the uncontrolled fermentation of milk, is furnished by the following references: Leichmann (1894, 1896), Aderhold (1899), Severin, Wolff, Heinemann (1906), Kruse (1903, 1910), Weigmann (1910), (Further controversies also arose in studies of the stereochemistry of soured milk. See references under "Stereochemical Lactic Acid Fermentation.")

#### b. Identity and nomenclature.

Considerable doubt still exists concerning the exact identity of the organism isolated by Hueppe. It was soon shown that it at least closely resembled, and was probably identical with Escherich's *Bact. lactis aerogenes* which this investigator isolated from the stool of a suckling infant and from uncooked cow's milk. The present tendency is to do away entirely with the term *B. acidilactici* and to apply the name *B. aerogenes* to the type species of this group. Probably a better procedure, (under the influence of workers in the Dairy Division), is simply to refer to it as a "high ratio" member of the *coloh-aerogenes* group.

### 3. Salient characters common to the group.

#### Morphology:

Thick, short rods.  
Gram negative.  
No spores.

#### Physiology: (See also following chapter).

Optimum temperature, 35° C. to 40° C.;  
between those of the other two most common  
groups of lactic acid bacteria.

Grow better in the usual laboratory media  
than the second and third groups do.

Milk is coagulated; curd is hard and not  
homogeneous, tends to express whey.

Optimum oxygen concentration is higher  
(usually) than for second and third groups.  
"Facultative anaerobes".

Produce gas from fermentable carbohydrates,  
and a large per cent of volatile acids. This  
group possesses several enzymes acting on the  
carbohydrate substrate and do not set up "true"  
lactic acid fermentation. In many agricultural  
lactic acid fermentations, the presence of some  
of these products is undesirable. This, and  
in some cases, their source, is responsible for  
the term often applied to them --- "undesirable  
lactic acid bacteria".

### 4. Sub-groups and distinctions between members of the group.

Probably upon no other question in microbiology  
has there been more work done than upon the diagnostic char-  
acters distinguishing between the members of this group of  
lactic acid bacteria --- the colon aerogenes group of the  
sanitary bacteriologist.

Many elaborate systems of sub-dividing this  
group have been made. In the preliminary preparation of  
this paper these were reviewed. Since then, this has been  
more satisfactorily accomplished by an exhaustive review  
of the classification of the whole colon-typhoid group  
made by Winslow, Kligler and Rothberg (1919). (This re-  
ference will furnish a review of the systematics of that  
larger group.)

a. Means of differentiation used by  
the early workers.

Beginning with the work of Smith, the most important distinctions have been made upon the type of fermentation induced by the different members, as indicated by the substances attacked and by the products formed. Until recent years most attention was paid to qualitative differences in the fermentations of these organisms, such as the power to ferment different carbohydrates with production of acid or of acid and gas, or of other products, as used in the Vosges-Proskauer reaction.\* This led to systems of classification such as those of MacConkey and others.

Following this, attempts were made to show quantitative differences in the products formed, such as amount of titratable acidity and percentage of gas produced. With the methods then used, such determinations are very difficult and the results obtained gave classifications only of temporary value. It was not until more recent times and by use of the refined technique of the physical chemist that a system of further dividing the colon aerogenes group was put upon a firm basis. The most important bases of differentiation used are the "gas ratio" and hydrogen ion concentration produced in lactic acid fermentations induced by these micro-organisms.

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\* See "Other Products" --- Glycols.



b. "Gas ratio".

In the earlier work on the classification of the group of colon-like micro-organisms, attention was paid to quantitative differences in the amount of gas produced, as measured by the Smith fermentation tube. Slightly later attempts were made by the same method to show quantitative differences between the ratio of carbon dioxide and hydrogen produced by the colon-aerogenes group.

It was soon shown, however, that such determinations were not absolute and were open to errors vitiating the results so obtained. Harden (1901, 1905) studied the ratio of these gases by chemical methods of gas analysis (which had actually been used by the earliest workers on gaseous fermentations, even before the introduction of the fermentation tube). He found that *B. coli* produced carbon dioxide and hydrogen in practically equal amounts, while the *B. aerogenes* type produced higher ratios of carbon dioxide. Keyes, (1909), and Keyes and Gillespie, (1913), made further studies by similar methods. Later, Rogers, Clark and associates investigated with refined methods the ratio of the gases produced during the strictly anaerobic fermentation of glucose by a large number of strains of both of these types. They found that most of the strains tested could be grouped into classes --- those

in which the gas ratio was  $\frac{1.06}{0.009}$ , and those in whose fermentations the gas ratio produced was  $\frac{1.90}{3.00}$ . In their report of these investigations, they show that this grouping has a significant relation to the source of the organisms. This work placed the "gas ratio" as a valuable diagnostic character in determining subgroups within the "acid gas" group of lactic acid bacteria.

c. Hydrogen ion concentration reached in defined systems.

Clark and Lubs devised another character of value in differentiating within the "acid gas" group. They found that in certain glucose media the final hydrogen ion concentration reached by organisms of the "low" and "high ratio" groups diverged to pH<sup>t</sup> zones which could easily<sup>be</sup> differentiated by use of the indicator methyl red. This means of subdividing the *colon aerogenes* group is known as the methyl red test and is discussed later in this paper (see "Theoretical Progress of Lactic Acid Fermentation"). It is a test more within the reach of equipment of the usual laboratory than are determinations of the gas ratio.

d. Correlation of tests.

Besides the correlation above shown to exist between the "methyl red" and "gas ratio" determinations, Levine, Hulton, and others have shown a farther correlation between them and the older Voges-

Proskauer reaction. In view of the striking relationship existing between these tests and apparently also with the source of the colon-aerogenes or acid gas group of lactic acid bacteria, it seems most logical to make our sub-division of this group of lactic acid bacteria upon that basis into "high ratio", ("methyl red negative", Vooges-Proskauer positive), B. aerogenes group and the "low ratio", ("methyl red positive" or B. coli)group. In the present status of our knowledge of these lactic acid bacteria, this subdivision is all that can safely be made. Furthermore, it adequately serves the purpose in a discussion of the lactic acid bacteria from the standpoint of agricultural lactic acid fermentations.

e. Fundamental basis of these tests.

In a study of lactic acid fermentation the fundamental basis of the above tests is very evident. All of them depend upon the progress and products of the energy-obtaining reactions of these lactic acid bacteria. By imposing definite conditions, it has been possible to control the rate and direction of these fundamental processes as well as the secondary reactions upon the products of the fermentations characteristic of the different groups.

5. Distribution and source of lactic acid bacteria of this group.

a. Distribution.

The members of the acid gas group are widely distributed and appear in the natural flora of most of the media of agricultural lactic acid fermentations. Besides their presence in the intestines of warm-blooded animals, they are found in milk, the upper layers of soil and on the surfaces of plants.

Largely due to the accepted intestinal and fecal habitat of the best known member of the group, their presence in food substances as named above was formerly attributed merely to direct contamination with feces or to fecal matter carried as dust. Under this assumption, all the members of this group were considered to be from one identical source, the feces of man or of other animals.

b. Source.

The source of the members of the acid gas group is of utmost importance in sanitary aspects; hence, it is evident that any subdivision possible to make, dividing the group upon that basis, would be of significant value. With this in view, workers in the Dairy Department of the Bureau of Agricultural Industry investigated a large number of cultures of members of this group



obtained from many sources. As a result of their work on acid gas bacteria from bovines, they state: "Colon bacteria of the bovine intestine belong, apparently without exception, to a single sharply defined type characterized by production of carbon dioxide and hydrogen in the ratio 1:1.06 ." (Rogers, Clark and Evans). Further work revealed that this relation is not so absolute in the case of acid gas bacteria from human feces (Rogers, Clark and Lubs, 1918).

However, it may be regarded as established that "low ratio" organisms are usually from feces.

The source of the "high ratio" group seems to be more diverse. They have been isolated from grains of various kinds, (Rogers, Clark and Evans), from silage and growing fields of alfalfa and kefir (Hunter), and from soil, (Johnson and Levine). This probably explains the not infrequent reports by earlier workers of the presence of bacteria indistinguishable, (by methods then in use), from *B. coli*, on hay and dried grains (Prescott (1903) and others). It is quite possible that their natural habitat is the soil.

#### c. Significance of presence.

The significance of the presence of acid gas bacteria has been materially modified by recognition of the sub-groups above established. No longer

can the presence of these organisms be attributed absolutely to fecal contamination. The tests above are of importance to the sanitary bacteriologist as an aid to the interpretation of the presence of acid gas fermenters of lactose. They are not, however, absolute in the case of organisms from human feces.

This paper is more directly concerned with the significance of their presence in the media of agricultural lactic acid fermentation.

Levine concludes that, in view of the fact "that members of the acid gas group are not uncommon on grains, their presence in food substances should not be regarded as conclusive evidence of sewage pollution."

Ayers and Clemmens (1916) have reported on the significance of the members of this group in milk.

They point out that the colon count, as made, includes both the *E. coli* and *B. aerogenes* types. As these types are usually of different source, such counts can never be a direct measure of manurial contamination. "Fresh milk produced under the best conditions always contains some organisms of the colon-aerogenes group", but rarely in large numbers, even when produced under the worst conditions usually encountered. "In fresh milk the colon count does not indicate the extent of direct manurial contamination, but does indicate the general conditions of cleanliness under which the milk was produced." Due to temperature relations of this group, high colon counts are usually indicative of improper holding of the milk. Although they did not find the differentiation of *B. coli* and *B. aerogenes* of material value in tracing the conditions of milk value, they suggest that more extensive studies may show it to be of value.

Finkelstein (1919) has also reported on the significance of the presence of the colon group in milk.

#### IV. Strep. Lacticus Group.

##### 1. Microorganisms included.

This group of microorganisms is sometimes called the Lactococcus group (Beierjink), or the Bact. lactis acidii group, but is usually known as the Strep. lacticus group. It is exceedingly difficult to define the limits of this group of lactic acid bacteria. Löhnis apparently has established the group in his system upon a purely morphological basis. He included only streptococci in the group, but has made it embrace practically every type of streptococcus that was known at that time. If lactic acid bacteria must be divided into groups and systems, it is doubtful if morphology is the logical basis.

Some observers, especially in the earlier days, claimed that even the more common members of the group were rods. Although at times some of them appear as rods, those most frequently encountered in the lactic fermentation of milk are distinct streptococci. Batson (1909) pointed out that "forms exist running all the way from a true streptococcus to a bacterium proper" but that "the weight of evidence is largely on the side of its streptococcal character". It is probably of little importance here whether they are rods or streptococci. If the grouping is made upon a physiological basis as to the type of lactic fermentation induced, a number of rod forms would probably be included.

Another source of error following the establishment of groups of lactic acid bacteria upon

As an example of close relationship existing between organisms of this group that are distinct streptococci with others that are unquestionably bacilli, may be mentioned Roger's motile bacillus which was pronounced a typical Strep. Lacticus when examined by Heinemann.

a morphological and generic basis, is the fact that all streptococci most certainly do not produce the type of lactic acid fermentation usually assigned to this group. Certain acid producing streptococci found in cheese do not seem to yield any lactic acid at all among their acid products. (Evans, Hastings and Hart) Again, the inclusion of the gas producing streptococci of Löffler (altho' they do produce lactic acid) will require further extension of the usual definition of the boundaries of the group.

There is little definite knowledge at hand concerning the type of lactic acid fermentation brought about by a number of lactic acid producing microorganisms which may be included in this group of lactic acid bacteria. It is probable that upon a physiological basis, the pneumococcus and many different types of streptococci should be placed within the same large group of lactic acid bacteria which includes the lactic streptococci of dairy fermentations.

The dilemma in which one is placed in the assignment of many of these to "types" of lactic acid bacteria is indicative of the obvious lack of data to use in the grouping of lactic acid bacteria as such.

## 2. Type species.

### a. History.

The type species commonly assigned to this group is probably identical with, or at least very



closely related to, the organisms isolated by Leichmann, by Waten, and by Günther and Thierfelder. (See "History"). In the confused terminology of the earlier periods, various names have been applied to this type of lactic acid bacteria. The term in most general usage, Strept. lacticus, was introduced by Kruse in 1903.

The earlier history of the type member of this group involved several years of bitter disputes over its relation to Huemke's acid gas bacillus, and over the part played by these two types of lactic bacteria in the natural souring of milk. (References to the literature on these questions have been given in the discussion of the type species of the preceding group. (paragraph III 2. a., of this chapter.)). The settlement of these questions established a distinction between these two types of lactic organisms. They also showed that the Strept. lacticus type was probably the more important agent in the natural souring of milk.

In the earlier days these lactic organisms were usually considered as rods. Kruse in 1903 pointed out the fact that they were probably streptococci. Experimental work by Helling, Reichenow, Salts and others confirmed Kruse's contention.

The recognition of the frequency of occurrence of these organisms in milk, and of their importance as

the usual agents of dairy lactic acid fermentations, gave peculiar significance to the claim that these organisms were streptococci.

At about this time the public health and sanitary significance of the presence of streptococci in milk was a moot question. Many authorities were describing a causal relationship between the streptococci of milk and various diseases. The normal occurrence of harmless streptococci in milk was not recognized in the earlier investigations and reports on the frequency of streptococci in milk foods (Peterson and others). It is obvious that the establishment of the streptococcal character of these lactic organisms of sour milk would place them in the very midst of their dominant group as one of the sanitary aspect of streptococci in dairy products. Controversies naturally followed over the possible relationship of these lactic acid bacteria to the streptococci which were recognized as disease producing organisms. This work probably had much to do with the present more exact interpretation of the public health significance of streptococci in dairy foods.

The latter history of the type species of this group includes the attempts to determine the relation of this type of lactic acid producing streptococci to other streptococci. The importance of this relation is obvious. It is reviewed in detail in Part II of this thesis.

### b. Identity and nomenclature.

The application of the generic term of "Streptococcus" to any group or type of microorganism is never an auspicious introduction to an establishment of its identity and nomenclature. Altho' the identity and nomenclature of strep. lactis are in a most chaotic state, it is no more pronounced than are those of most of the other proposed types of streptococci.

The strep. lactis type of streptococcus is encountered in the investigations of agricultural, sanitary and medical bacteriologists. These workers

have had different objects in mind and the application of different tests and methods of study to the investigation of the lactic streptococci have furnished much valuable knowledge of the group. On the other hand, the over emphasis of certain characters and the neglect of others by the workers in the different fields have led to a great deal of confusion which makes very difficult any statement on the present status of the identity of the Strept. lacticus of dairy lactic fermentation.

The importance of this question has led to its experimental investigation in Part II of this thesis.

### 3. Salient characters of the group.

The characters of the lactic group of streptococci are reviewed in detail in Part II of this thesis. These the characters usually assigned to these streptococci are compared with the characters exhibited by a number of strains of streptococci isolated from sour milk, sour cream, and from the cells of other agricultural lactic fermentations.

A more extensive treatment of this topic will be found in that place.

#### Morphology:

Most members of the Strept. lacticus are oval in form, closely resembling some types of the pneumococci; oftentimes occur in chains, especially in sugar or serum broth.

Gram positive; no spores.

Sometimes with capsules. Fensholt (1918) states that most lactic acid bacteria form capsules in milk cultures during the younger stages of their life history; he believes their ability to become slime formers is due to this property. At the time of curdling, the capsules are said to disappear, and with them the slimy condition of the milk. (Gorini (1918) makes a similar statement.)

#### Physiology: #

Optimum temperatures: 20° to 35°C; usually lower than for most members of the preceding group. Low minimum temperature.

Apparently grow as well under low oxygen concentration, as under aerobic conditions.

Set up a "true" lactic acid fermentation; lactic acid is the only product produced from sugars, in large amounts. No gas; very slight amount of volatile acids produced by the organisms usually assigned to this group.

Most members of the group coagulate milk. The curd produced is smooth and homogenous, and upon stirring it can usually be reduced to the original colloid condition. There is little tendency for the curd to express whey. The behavior of litmus milk cultures is considered characteristic by many observers.

#### 4. Differentiation of the Strep. lacticus of dairy fermentations, from other types of streptococci.

a. In view of the fact that such a close relationship has been shown to exist between Strep. lacticus and other streptococci, it is evident that their differentiation would be difficult. Upon this subject, Heinemann (1906) has made the following statement: "A careful perusal of von Linselsheim's summary of characters of streptococci demonstrates conclusively that there is no salient

# Further facts of interest in relation to the physiological characters of the different groups of lactic acid bacteria are furnished in a following chapter. ("Influence of environment upon the Lactic Acid Bacteria".)



difference between recognized streptococci and lactic acid bacteria of the Leichmann type.... Strep. lacticus agrees in morphological, cultural and coagulative powers with pathogenic, fecal and sewage streptococci". Kruse makes a similar statement. Descriptions of the lactic acid streptococci given in the older systematic bacteriologies, such as those of Wigula, Chester, and others, could be applied with equal fitness to the streptococci of pathogenic and fecal origin. The work of Rogers and Dahlberg, of Sherman and Hastings, and of Evans (1916), showed a close agreement between the morphological and cultural characters of the lactic streptococci and those of the streptococci of mastitis and other pathological conditions.

The importance of such differentiations is obvious. They are essential for the interpretation of the rôle of different streptococci in agricultural and other processes; they are of the utmost significance from a public health standpoint as a means of distinguishing between the harmful and beneficial streptococci of dairy products. The importance of comparing different means of differentiating the lactic streptococci from other types of streptococci has led to the investigation reported in Part II of this paper. There the characters of a number of lactic streptococci from fermented dairy products are compared with the characters of streptococci from human pathological conditions, from bovine mastitis, from certain cheeses, and from sauerkraut. A more extended discussion of the differentiation of lactic streptococci is furnished there.

5. Distribution and source of lactic acid bacteria of this group.

a. Distribution and source of the Strept. lacticus or sour milk type of streptococci.

Streptococci of this type are practically always present in milk and in milk products. Their relative numbers rapidly increase during the natural souring of milk, to such an extent that they are the dominant type in the uncontrolled fermentations of milk and cream. Moreover, in modern dairy practices, large numbers of the lactic streptococci are added to raw or pasteurized milk or cream, in the form of so-called "starters", as a means of obtaining the type of lactic fermentation desired in certain dairy products. Hence, the lactic type of streptococci are usually found to be dominant in such products as sour milk and cream, butter, some fermented milk drinks, and certain cheeses, - whether these products are the result of the controlled or of the "natural" or uncontrolled fermentation of milk or cream.

It is impossible to state whether or not the Strept. lacticus type of lactic acid bacteria are distributed widely in nature. The investigation of Eaton would indicate that the occurrence of the typical Strept. lacticus is rare except in the environs of the dairy. Other workers report the presence of similar

organisms on plants and various plant products. However, many of the lactic organisms described as typical agents of the lactic acid fermentation of plant foods, do not resemble very closely the typical Strep. lacticus of sour milk. (See Part II for criticism of Kruse' interpretation of the wide distribution of Strep. lacticus.) It is probable that "Strep. lacticus is a normal inhabitant of the intestines and saliva of animals". (Warr, 1916)

The source of <sup>the</sup> Strep. lacticus found in milk foods is an important but a difficultly solved question. The results of numbers of investigations make it doubtful if the udder of the cow is an important source. Eaten came to the conclusion that the mouth and saliva of the cow were the most important foci of dissemination of the lactic streptococci found in milk. As secondary sources, Eaten mentions mangere, body surfaces of the cow, "any material that is within reach of a cow's mouth", unclean utensils, etc. Other investigators (Warr, Rogers and Dahlberg, and others), are less willing to dispose of the cow's feces as an important primary source of the common lactic streptococcus.

It is obvious that the interpretation of the distribution and source of the lactic type of streptococci must depend entirely upon the interpretation of the boundaries of the so-called Strep. lacticus group. This point is again emphasized in Part II of this thesis.

b. Distribution and source of other members of the group, with special reference to their occurrence in milk and milk products.

This question, of course, is dependent entirely upon what other lactic acid bacteria are included in this group. If one includes the lactic bacteria reported by Weiss, Spatein, Rutjagin, and other investigators of fermented plant foods, the wide distribution of these lactic organisms must be considered. However, altho' Kruse interprets these as typical lactic streptococci, there really is too little data to warrant placing these lactic acid bacteria in any particular group. Apparently there are a large number of lactic acid bacteria commonly found in fermented plant products, which prefer sucrose as a source of energy. (Such a streptococcus from sauerkraut is included in the investigations reported in Parts II and III of this thesis.)

The fact that the streptococci of human pathological conditions, of bovine mastitis, and still other types of streptococci are closely related to the lactic streptococcus, requires a brief consideration of the distribution and source of these organisms. The following discussion is limited to their occurrence in milk and milk products.

The dominant microbial type in sour milk, is the lactic type. However, in milk in the earliest periods of its handling, the stren. lacticus type has not become dominant. In fact, in milk drawn from the udder with aseptic precautions, other types of streptococci predominate. These are usually considered to be of udder origin and are sometimes associated with bovine mastitis. Examinations of very fresh milk (especially certified milk or milk which has been free from the usual outside contaminations) will reveal considerably larger relative numbers of such streptococci than will milk which has begun to sour. On the other hand, other types of streptococci (such as R. C. Avery's hemolytic cheese strains) may be found only with difficulty in fresh milk, and yet be present in significant numbers in certain dairy products.



Such changes in the relative numbers of the different types of streptococci in milk and milk products are due largely to the influence of different environmental conditions upon the life processes of the different organisms. The fitness of the environments obtaining in milk, and in milk products at the different periods and stages of their manufacture and storage, will determine the survival and relative numbers of the different types of lactic acid bacteria which may be introduced into these systems.

(The importance of this relation suggested the study of the relative influence of different environmental conditions upon the life processes of different types of streptococci. This furnishes the basis of the investigation reported in Section A of Part III of this thesis.)

## M. Lactobacillus Group.

### 1. Micro-organisms included.

This group is usually known as the Lactobacillus Group, or the *B. bulgaricus* or *Bact. caucasicum* Group. Besides the *bulgaricus* bacillus and its closer relatives, the lactobacilli include *B. delbrückii* and many other lactic acid bacteria of the brewery and distillery and also the so-called Boas-Oppler bacillus. Recent work by Fred and associates indicates that still other types must be recognized.

### 2. Type Organism.

*Lactobacillus bulgaricus* may be taken as the type organism of this group. The identity of even the type species is not definitely established and different names are frequently applied to it. Makrinoff and others believe that many of the organisms to which various names have been applied by different authorities are identical with the *bulgaricus* bacillus.

### 3. Sub-groups and distinctions within the group.

#### a. Types recognized.

The group of lactobacilli includes a large number of organisms, many of which are very closely related. Attempts at subdivision of this group differ with the strains and types of organisms studied by the investigators. Apparently the lacto-

bacilli represent a much more extensive group than was at first recognized. Further work will probably make this still more apparent.

It has been claimed that *Bac. delbrückii*, *B. acidificans longissimus*, and most other lactobacilli of importance in the brewery and distillery ferment maltose but not lactose, (compare Hennenberg), and may in this way be distinguished from the lactose fermenting *B. bulgaricus* type. The pentose fermenters of Fred, however, seem to represent hitherto unrecognized but important members of the lactobacillus group.

Most of the other lactobacilli recognized in the earlier attempts at subdivision of this group have been types more closely associated with the dairy industry. These seem to be closely related among themselves.

#### b. Systems proposed.

Löhnis and White and Avery attempted to separate the lactobacilli studied by them into two groups upon the basis of presence or absence of granules, degree of acid production and optical form of the lactic acid produced. Such a division, however, is open to criticism; granule production is not a very independent character; later work has also shown that within the lactobacillus group are included organisms that produce all modifications of lactic acid. The degree of acid production seems to be a more constant

factor and it is possible that a division can be made in the future upon the basis of hydrogen ion concentration into a "high acid" and "low acid" group.

In his 1910 system, Löhnis recognizes six types, based upon a variety of characters. This classification is perhaps too unwieldy for use. Moreover, it seems that many of his types are too closely related to warrant separate treatment.

White and Avery's classification is to be preferred to that of Löhnis, largely because it is simpler and does not introduce a confusing number of closely related types. Later work, however, has shown that their system, while holding for the types then recognized, would be of little value in the distinction of all of the now recognized types.

More recently, Rahe has proposed a classification based upon fermentation of carbohydrates. His classifications, however, will also likely prove of only temporary value.

Jensen (1919), recognized a larger number of types in his classification. He bases his two genera of such forms upon modification of lactic acid produced, fermentation reactions and temperature relations. His system is of little value as a system, although the descriptions of the types are a valuable contribution.



Fred's pentose fermenters introduce further complications to any attempt at a workable but inclusive system of sub-grouping the lactobacilli.

c. Probable inadvisability of subdivision of the group.

In the present state of our knowledge of the lactobacilli, it is perhaps best, in a study of agricultural lactic acid fermentation, to attempt no subdivision of the group. Although many slightly differing species or strains may be included, Barthel suggests that they may best be considered under the general group name, as in the usual treatment of the *Strep. lacticus* group.

If a system of sub-grouping is made, it should be upon the fundamental and predominating metabolic process of the lactobacilli --- the acid fermentation of carbohydrates. Upon that basis, future work may divide them into "high acid" and "low acid" groups with possible subdivisions on the amounts of other acids (besides lactic acid) produced.

4. Salient characters of the group.

a. Morphology.

The lactobacilli conform more or less closely to the following salient characters of the group:

Usually long rods, varying widely in form and size; in older cultures there is a decided tendency to filament formation; Y-shaped forms sometimes appear.

Gram positive, although this is not a constant character; often part of a rod will retain the stain, while the rest of it will be decolorized. Often granulated; upon staining with methylene blue or with a granule stain, such as Weisser's, the granules may be distinctly demonstrated.

No spores; capsules rare.

Most members of the group are non-motile, although flagellated forms have been reported by several investigators.

#### b. Cultural characters.

Optimum temperature, 40° C. to 50° C., although one of Jensen's genera has a maximum temperature of 35-40°C.

Usually scanty growth on routine laboratory media. This has occasioned considerable difficulty in the cultivation and isolation of certain lactobacilli (see later).

Agar colonies have much the same appearance as those of *Strep. lacticus*, with the exception that they have a more radiating "tangled hair" appearance.

#### c. Physiology.

Optimum oxygen concentration is much as for the preceding group.

The lactobacilli are usually considered as "true" lactic acid bacteria, producing only traces of

other acids than lactic acid. This is not at all absolute and varying amounts of other products are frequently formed. The production of significant amounts of volatile acid has been reported by a number of investigators (White and Avery, Heinemann and Hefferan, Bertrand and associates, Hart, Hastings, Flint and Evans).

Certain amounts of succinic acid are also often produced by strains important in the dairy (Currie, Bertrand and associates). White and Avery also report formation of small amounts of alcohol. Fred's pentose fermenters form large amounts of acetic acid. (See "Chemical Changes" and "Other Products".)

Even in milk, these organisms often grow more or less slowly. The curd produced is usually homogeneous and easily broken up. The presence of casein dissolving enzymes has been demonstrated in some of the lactobacilli.

#### 5. Distribution and source.

*Bac. delbrückii* and similar lactobacilli were known for a long time in connection with the brewing and distilling industries. Other members of the group were isolated from cheese by Adametz and by Von Freudenreich. A little later, *B. bulgaricus* or very similar lactobacilli were isolated from fermented milk drinks and received considerable study (Rist and Khoury, (1902), Duggeli (1906), Cohendy (1906), Kuntze (1909), Leva). At that time these micro-organisms were not

considered to be widely distributed, but later investigation has shown them to be of general occurrence.

Leichmann, in his early work on the bacteria responsible for the souring of milk, described a thermophilic rod form which was probably a member of this group. Since then they have been found to be widely distributed in milk and milk products, (Hastings and Hammer (1909) and others). They were isolated from American cheeses (Evans, Hastings and Hart (1914), Eldredge and Rogers (1914) and others); from pasteurized whey (Dotterer and Breed); from ensilage (Hunter, Sherman); from sauerkraut and other fermented foods (Heinemann and Hefferan).

Other lactobacilli, probably closely related to those of agricultural lactic acid fermentation, have been isolated from gastric fluid of patients suffering from carcinoma of the stomach (Heinemann and Ecker). It is also thought possible that the so-called *Leptothrix buccalis*, sometimes associated with dental caries is a lactobacillus of the *bulgaricus* group. (A discussion of such lactobacilli from the standpoint of the medical bacteriologist is furnished by above references of Heinemann and Ecker, and Rahe).

Their ubiquity was summed up by Heinemann and Hefferan who found them "widely distributed in nature, occurring normally in human feces, in the feces of cows and horses, also in a variety of sour and aromatic foods, in food for cattle, in normal gastric juice, in various fermented milks, in ordinary market milk and in soil, both manured and not manured."

Although these organisms seem to possess a higher temperature optimum than that ordinarily prevailing in the outside world, Lohnis, (1912), believes it



probable that they can adapt themselves to other conditions so as to exist and even multiply at the lower temperatures prevailing in soil and in other of the above mentioned environments.

It has been shown that the lactobacilli are widely distributed. They probably are present, at least in small numbers, in the media of most agricultural lactic acid fermentations. However, only if the prevailing conditions are favorable for their development in the struggle for existence among the natural flora of the medium, will they become dominant.

It is probable that, as found in agricultural lactic acid fermentations, they are of soil or fecal origin. Stevenson concluded that their natural habitat is the alimentary tract of animals. Here they would find optimum conditions --- "high temperature, low oxygen concentration and association with other microbes." The intestines of animals would indeed furnish an ideal focus from which these organisms could be distributed to the many sources from which they have been isolated.

#### 6. Cultivation and isolation of lactobacilli.

As considerable difficulty is sometimes encountered in the isolation and cultivation of some of these organisms on the usual culture media, a short discussion of methods used by different investigators may be of interest.

### Cultivation:

In their review of the value of different media in the cultivation of lactobacilli, Bertrand and Duchacek describe the media tested by them as follows:

#### Excellent:

Milk and calcium carbonate.

Milk alone.

Medium consisting of,

{ 30 gms. malt boiled for 15 min. in  
1 liter of water.

{ 1 % peptone

{ 3 % precipitated calcium carbonate

{ 4 % fermentable sugar

#### Fair:

Medium consisting of,

{ yeast extract

{ peptone

{ calcium carbonate

{ lactose

Milk serum and calcium carbonate.

Wort and calcium carbonate.

#### Poor:

Malt )

or ) + peptone and lactose, but

Yeast) - calcium carbonate.

Milk serum + peptone, but - calcium carbonate.

White and Avery and others found whey agar a favorable solid medium.

Many authorities claim that these lactobacilli grow only with difficulty upon the usual meat-peptone media. Rahe, however, reports good development of cultures on such a medium, (with a fermentable sugar), if the "natural acidity" of the broth constituents is not disturbed by neutralization. He ascribes this to a favorable influence of certain unaltered nitrogenous constituents --- "amino acids". From work done on pH requirements of lactobacilli, it would seem that this may have been due simply to the hydrogen ion concentration of the medium.

Enrichment methods are usually employed, by the addition of such nitrogen and carbohydrate substances as are found in milk or whey, peptone, yeast extract or malt, and a fermentable sugar, often lactose. (White and Avery, however, consider dextrose more favorable.) The beneficial effect of a highly buffered medium is obvious.

#### Isolation:

For isolation of these organisms it is often necessary to resort to selective methods, as other micro-organisms are usually present in large numbers, together with the lactobacilli.

The first step in the usual procedure consists in placing the substance from which isolation is to be made under conditions tending to increase the relative number of the lactobacilli and, if possible, to eliminate most of the other micro-organisms. To do this, advantage may be taken of the high optimum temperature and hydrogen ion concentration toleration of the lactobacilli. To isolate them from milk, Hastings and Hammer incubated the milk at high temperature until maximum acidity was reached and then transferred to new medium. In this way most of the other lactics are eliminated, although some yeasts persist. As a selective medium, Leva used extract agar + .35% lactic acid, Heine-mann and Hefferan used dextrose broth + .5% glacial acetic acid, Rahe used acetic dextrose broth of n/20 normality.

By the use of such selective media, cultures may be obtained which contain the lactobacilli, if not in pure culture, at least in predominance. The isolation then would consist in plating them out on a medium suitable for growth.

#### W. Fourth Group of Lactic Acid Bacteria.

##### 1. Micro-organisms included.

As mentioned before, it is this group which offers the greatest difficulty to those trying to set up a system of lactic acid bacteria. In this group it is

especially evident that many lactic acid bacteria have little in common beyond inability to produce lactic acid from sugars. The third group of Rogers and Davis is quite similar to this group in Löhnis' system. In place of this group, Kruse (1910) proposes the "Acid-lab" group, in which he places the "acid-lab" organisms of Gorini and many other bacteria not usually considered as lactic acid bacteria, (although they do produce a certain amount of lactic acid), such as *Proteus vulgaris*, *B. prodigiosus*, and possibly some spore bearers.

The best treatment, perhaps, is to consider as belonging to this group lactic acid producing bacteria, mostly micrococci; most of which exhibit a low minimum temperature, many of them with distinct proteolytic powers.

Among the diversity of quite different species collected within this group, the most common forms are such species as *M. lactis acidii* and *Staph. pyogenes*.

## 2. Salient characters.

The lactic acid bacteria of this group represent rather a heterogeneous collection. Not all of them will conform to the following salient characters of the more common members.

### a. Morphology.

According to Löhnis, this group is limited to micrococci which come singly or in groupings different from streptococci. In a review of his work, Gorini (1915) emphasizes the purely physiological basis



upon which he has established his "acid-lab" group. He includes rod forms as well as cocci. Recent work by R. C. Avery suggests that certain streptococci would also fall into this group. Bockhout and deVries' cheese bacterium also possesses the physiological characters of the "acid-lab" group.

### b. Physiology.

Optimum temperature of many forms is the lowest of all the groups, at or below  $20^{\circ}\text{C}.$ ; of other forms,  $37^{\circ}\text{C}.$ ; minimum temperature is below  $10^{\circ}\text{C}.$  for many species. Usual growth on laboratory media may be slow, but final growth is usually more luxuriant than in the two preceding groups. Nitrate reduction is commonly exhibited. The products of fermentation of the members of this group differ greatly and have not been investigated so thoroughly as the preceding groups.

### 3. Distribution and Source.

These lactic acid bacteria, according to Löhnis, are fairly common in milk kept 8 to 14 days at a low temperature ( $2^{\circ}\text{C}.$  -  $5^{\circ}\text{C}.$ ). Gorini reports that they are often present in butter or cheese kept in cold storage. Evans found them common members of the so-called udder flora. In their investigation of the udder flora, Harding and Wilson found that 70% of the cultures isolated formed lactic acid from lactose; many of these apparently belong to Gorini's group. Aderhold found acid-lab lactic bacteria in fermenting beans. Sayre, Rahn and Farrand found members of this group among the most frequent micro-organisms present in butter stored at low temperatures. R. C. Avery has found similar organisms quite commonly present in various kinds of cheese.

## VII Other Lactic Acid Producing Bacteria.

The above discussed groups, although including most of the bacteria responsible for agricultural lactic acid fermentation, do not include all of the bacteria which produce lactic acid. Many others have been shown to form considerable amounts of this acid.

Lactic acid fermentation is frequent among vibrios (Peran, Hanan and Huyse, Gosio, Kuprianow and others). It is also reported in the case of diphtheria bacillus (Dziergowski) and a few spore bearing bacilli. A still larger number form small amounts of lactic acid. In fact, Benecke believes it probable that all bacteria produce a certain small amount.

These micro-organisms are not important as agents of lactic acid fermentation. It is interesting to note, however, that much work had been done on lactic acid production by such bacteria, especially the vibrios, several years before the isolation and recognition of the most common agent of lactic acid fermentation.

## VIII Lactic Acid Production by Other Organisms.

In our preceding discussion, it is seen that bacteria from almost all genera are capable of lactic acid fermentation. Although it is with lactic

acid bacteria that the agricultural examples of lactic acid fermentation are chiefly concerned, many other micro-organisms have been shown to produce certain amounts of lactic acid.

It is apparently lacking in actinomycetes, (Kruse), and rare among the yeasts, (Löhnis, 1910, Buchner and Meisenheimer). Some molds possess the power of lactic acid fermentation; Saito has demonstrated it in the case of *Rhizopus chinensis*; Calmette and Boullanger, in the case of other hyphomycetes.

Biological production of lactic acid is, however, not limited to the action of micro-organisms. McGeorge and Habermann have detected lactic acid in the leaf juices and extracts of plants. The production of lactic acid in animal tissue has been known for a long time. (See under "Substrata of Enzymes"). In view of the work of Stoklasa, proving the formation of small amounts of lactic acid by the intramolecular respiration of green plant tissue in absence of air, it seems that lactic acid production in at least small amounts is a property common to protoplasm.

Stoklasa reports lactic acid production by an enzyme present in cow's milk preserved by antiseptics. Lane-Clayton questions his conclusion and calls attention to the possibility of the liberation of enzymes from bacteria killed by the chemicals added.

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D. BUREAU OF THE PACIFIC COAST SURVEY.

## ENZYMES OF THE LACTIC ACID BACTERIA

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## ENZYMES OF THE LACTIC ACID BACTERIA

### I. Proof of the Enzymatic Nature of the Lactic Acid Fermentation Process.

#### 1. Earliest demonstrations.

The proof of the enzymatic nature of lactic acid fermentation was not accomplished until the present century. The earlier attempts were made with methods applicable to extra-cellular enzymes, and yielded negative results, as the enzymes of the lactic acid bacteria are intra-cellular and seem to be retained jealously within the cell. Later attempts, with methods much the same as those used in obtaining the zymase of alcoholic fermentation, have been more successful.

In 1903, E. Buchner and J. Moisenheimer obtained, from cultures of *B. delbrücki*, a sterile dry powder, which formed lactic acid from sucrose. A little later, R. Herseg, working under Buchner's direction, obtained, by treatment of the cells of *B. acidilactici*, Hueppe (*B. aerogenes*), a sterile powder which formed lactic acid from lactose. These two investigations established the fact that lactic acid fermentation is due to enzymatic action, (or at least, that lactic acid may be produced from sugars, independent of the presence of living cells.)

It will be noted that the preparations of Buchner and of Herzog were derived from the cells of lactic acid bacteria of the first and third groups. Equally convincing results have not yet been obtained with the members of the *Strep. lactious* group, which includes the lactic acid bacteria probably most important in agricultural lactic acid fermentation. Here, the difficulty of obtaining an adequate mass of cells, and other factors, render such an attempt more difficult in the case of lactic acid bacteria of the second group. However, the experiments of Evans, Hastings and Hart "show that *Bact. lactic acid* is able to form acid in the absence of the living cell."

They assumed that cells killed by chemicals would undergo disintegration and liberate any enzyme present as well as though the cells were mechanically ruptured. To bring this about, they added an antiseptic to milk containing these lactic acid bacteria in active growing condition. They found that an increase in acidity of the milk occurred. Furthermore, the increase in acidity was in direct relation to the number of bacteria present in the milk at the time of adding the antiseptic. This strengthens the conclusion that the acid production is due to enzymatic action, as, if enzymes are operating, the acid production will be proportional to the amount of enzyme present, or to the mass of cells.

Although they did not isolate an enzyme from the cells themselves, they believed that their "results seemed to leave no doubt concerning the presence of an acid forming enzyme in organisms of the *Bact. lactic acid* (*Strep. lactious* Kruse) group, that act on milk sugar." It must be admitted, however, that, to date, no one has actually demonstra-

ted the production of lactic acid by "unorganized" material obtained from cells of members of the Strep. lacticus group. A more convincing demonstration than that above described would require a demonstration of production of lactic acid from a sugar in experiments controlled by cultural tests for absence of living cells, rather than by the use of antiseptics.

## 2. Later work on "acid gas" lactic acid fermentation.

The enzymatic relations involved in acid gas fermentation by *B. coli* have been extensively studied by Grey, as a continuation of the admirable researches of Harden and his associates upon fermentations of this type.

Grey, (1920), in a summary of his investigations, states the conditions which he considers necessary to establish the enzymatic nature of a fermentation process. He suggests that it is "not necessary to isolate from cells an unorganized material capable of bringing about a fermentation, in order to demonstrate that such a fermentation is brought about by enzymes (unless such substances were defined as enzymes, only provided they could be isolated by the present means at our disposal)."  
"If an enzyme is regarded as a substance capable of inducing fermentation independently of the life of the cell, then there are two methods of demonstration which serve to establish the existence of enzymes in any particular case, without the necessity of separating them from the cell: (1) carrying out the fermentation under conditions which do not support the life of the organism; (2) proof that the several fermentation phenomena are independent of one another, (for, if a series of functions of a cell are absolutely independent of one another, some of them, at least, cannot be essential to the life of the cell)."

Grey's method of attack was to test the action upon glucose and mannitol of salt solution suspensions of cells of *B. coli*. He divided the fermentation process into several phases upon the basis of

increase or decrease in the number of living cells. Determinations of the various products of the fermentation were made at different intervals and the courses of production of the different products were plotted. By this means, he fulfilled the conditions above prescribed by him, as he found: (1) the fermentation proceeded during the period of death of the cells; (2) the courses of production of various groups of products are different during different phases of the fermentation and under different conditions, and seem to be independent of one another.

Among his interesting findings, of particular moment here was the apparent independence of reactions, (mentioned above, see "Chemical Changes"), which go to produce different groups of products. The formation of lactic acid was conspicuous for its independence of the other reactions. During the phase of the fermentation immediately following the rapid multiplication of the cells, lactic acid was produced to the extent of 70% of the sugar consumed. During the period of death of the cells, no lactic acid was formed, although the glucose was consumed to a still greater extent than during the preceding phase. This period of death of cells was characterized by the transformation of the sugar to alcohol, formic, acetic, and succinic acids.

Grey admits that "the separation of the phases of the fermentation was not absolute, either as regards complete absence of living cells at any one time, or complete transformation of the glucose in one direction only." It seems, however, that he is well justified in his conclusion, that "taken in conjunction with the earlier work of Harden and Penfold, and later, of the writer, the present results leave little room for doubt that the several fermentation processes by which *B. coli communis* brings about the decomposition of glucose and allied substances are true enzyme actions and are capable of



acting independently of one another, and thus breaking down the sugar in various ways."

It is very possible that the enzyme which produces lactic acid in "true" lactic acid fermentation is the same as that involved in the production of lactic acid in "acid gas" lactic acid fermentation, and that the diversity of products in the latter type of fermentation is due merely to the presence of additional and independent enzymes.

## II. Correct Nomenclature for the Enzyme of the Lactic Acid Fermentation Reaction.

### 1. Objections to Buchner's "lactacidase."

Different names have been suggested for the enzyme responsible for lactic acid fermentation. "Lactacidase", suggested by Buchner, is probably in most general use. In many respects, the use of this name is unfortunate; it is not only contrary to the usual substrate nomenclature, but the same name is often applied, (perhaps more logically), to enzymes of other fermentation processes, such as those changing lactic acid to volatile acids, and it was also formerly applied to one of the assumed co-



## enzymes of alcoholic fermentation. #

# Many of the best authorities no longer consider lactic acid as an intermediate stage in alcoholic fermentation. Slator, as the result of velocity experiments, believes it "improbable that in alcoholic fermentation any but small amounts of sugar go through intermediate stage of lactic acid". Buchner and Heisenheimer (1910) found that "living yeast can neither form nor ferment lactic acid". The presence of lactic acid in wine is now ascribed to the fermentation of malic acid by bacteria. (Seifert and Rosenstiehl). While the above experiments indicate that at least most of the lactic acid found in wine is not derived by action of yeast upon sugar, they do not prove that lactic acid does not play a rôle as an intermediate substance in lactic acid fermentation.

Investigations with cell free yeast extracts present evidence suggesting the pertinence of Cohen's remark, which was reported in our discussion of intermediate substances in lactic acid fermentation. ("Chemical Changes"). H. Oppenheimer (1914) showed that lactic acid can be formed by cell free extracts of yeast (in experiments controlled by cultural tests for the absence of lactic acid forming bacteria). He believes that lactic acid is an intermediate stage in alcoholic fermentation; and points out that the reason lactic acid is not found in tests with living yeast may be due to the fact that the particular enzyme involved in the production of the lactic acid may possess greater resistance than do those involved in the production of alcohol. (The possibilities of such a relation are evident; they have been shown to exist in mixtures of other enzymes involved in similar reactions. E. g., the disaccharose hydrolyzing, and lactic acid producing enzymes of the pneumococcus. (See III 1. a. in this section)

Palladin and Sabinin (1916) found that killed yeast decomposed lactic acid in the presence of pyruvic aldehyde. (They admit two weaknesses in their line of evidence: (1) the formation of alcohol was accompanied by a relatively large yield of  $C_2$  and not in the ratio found in alcoholic fermentation; (2) killed yeast also decomposed a number of very dissimilar compounds, which are known to have no relation to alcoholic fermentation). They conclude: "Just as the negative results of Buchner's experiments do not prove that lactic acid can not be the intermediate product of alcoholic fermentation, our own positive results .... do not yet conclusively prove that lactic acid is an intermediate product."

## 2. Suggestions of Stoklasa, Czapek and others.

Stoklasa calls those enzymes producing intramolecular change in the glucose molecule "glykolytic" enzymes, and uses the specific term "lactolase" for the lactic acid producing enzyme. Malzevin used the term "pauteraze". These terms are still less in accord with accepted principles of enzyme nomenclature than Buchner's term. Czapek believes "glucolactacidase" to be a more fitting name.

## 3. Adoption of Euler's term -- "lactic acid bacteria zymase".

Probably the general term used by Euler is least open to criticism, from the standpoint that the exact substrate relation, as well as the number of enzymes concerned in lactic acid fermentation, is not definitely established. In view of our incomplete knowledge of these factors, it seems better to base our terminology upon the chemical reaction induced, rather than to use the usual substrate nomenclature. Upon this basis, Euler overcomes the above criticism, by applying to the enzymes or enzyme responsible for lactic acid fermentation the term "lactic acid bacteria zymase", in agreement with the use of "zymase" for "the sum total of the enzymes responsible for alcoholic fermentation."

Some of the champions of the entrance of intermediate stages into the process of lactic acid fermentation, have proposed names for the enzyme involved in the formation of lactic acid from the intermediate product. Dakin and Sudley (1913), who accept methyl glyoxal as an intermediate stage in the formation of lactic acid in the animal body, apply the term "glyoxalase" to the enzyme responsible for the change of this aldehyde to lactic acid. Neuberg and Kerb (1915) term the ferment changing methyl glyoxal to lactic acid, a "ketonaldohyde-mutase" (an extension of the term "aldohyde-mutase" given by Parnas (1910) for the enzyme serving in the Cannizzaro reaction of aldehydes.).

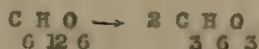
### III. Lactic Acid Producing Enzymes of Lactic Acid Bacteria.

#### 1. Nature of enzymes producing lactic acid from carbohydrates.

After the proof of the enzymatic nature of lactic acid fermentation, questions naturally arise concerning the nature of the enzyme concerned, the sequence of the reactions induced, and whether more than one enzyme is concerned in the process of producing lactic acid from sugars.

##### a. question of necessity of the presence of hydrolyzing enzymes.

Although the material extracted from the cells of lactic acid bacteria by Buchner and Heisenheimer and by Herzog produced lactic acid from the disaccharoses, sucrose and lactose, there is a certain tendency to assume that the enzyme directly concerned with production of lactic acid acts only upon hexoses, according to the usual formula,



In that case, other carbohydrates would have to be

changed by some means to that form, before being acted upon by the lactic acid bacteria zymase.\* This assumption, then, requires in lactic acid bacteria capable of lactic acid fermentation of polysaccharoses the presence of carbohydrate hydrolyzing enzymes to bring about this transformation.

Very definite statements are made by many authorities, (Oppenheimer, Bau, Wehmer, Rahn), to the effect that lactic acid bacteria that ferment disaccharoses always possess a hydrolyzing enzyme which first converts the disaccharose into its hexose components, before lactic acid fermentation takes place. It is probable that, at least in most cases, this hydrolysis does occur. The following paragraphs show, however, that there is little or no definitely supporting evidence afforded by investigation of lactic acid bacteria.

Certain authorities claim to have demonstrated the presence of such enzymes.

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\* The material used by the above investigators, then, would be a mixture of at least two enzymes, one of which was a carbohydrate hydrolyzing enzyme, and the other the true lactic acid enzyme. As a matter of fact, the presence of invertase has been reported in the case of the sucrose fermentation organism used by Buchner and Meisenheimer.



In his pioneer report, Hueppe (1884) claimed that his *B. acidilactici* fermented sucrose and lactose, only after hydrolysis. He reports the observation of a change in rotation of sucrose cultures. Hueppe's work can not be accepted as proof of the presence of hydrolyzing enzymes, altho' it is probable that his work furnished the basis of the empirical statements given on this question in the earlier text books.

Bertrand and co-workers claim to have proven an endocellular lactase in the case of *B. bulgaricus*. They state that lactose is by this means first converted into glucose and galactose, before production of lactic acid, and that failure of the lactobacillus to ferment maltose is due to its lack of the maltose enzyme. The demonstration of sucrase in the case of *B. delbrückii* (compare footnote, p. ) is reported by Kruse. Jensen, (1919), also believes that the lactic acid bacteria attack disaccharides by endocellular hydrolysis: "the enzymes which hydrolyze the disaccharides appear to be endoenzymes, and we must therefore suppose that these sugars are taken in (into the cell) as such." This authority claims to have observed the hydrolysis of lactose by old cultures of lactic acid bacteria.

It seems certain that, if lactic acid bacteria do possess enzymes hydrolyzing polysaccharoses, these must be endocellular. Although such enzymes are usually exocellular, certain other micro-organisms, as *Monilia candida* and some yeasts, have been shown to possess disaccharose splitting enzymes, which are strictly intracellular.

The evidence on the question of whether hydrolysis of a disaccharose must, in all cases, precede the lactic acid fermentation reaction is by no means all on the positive side.

Such an hypothesis would lead one to assume that the lactic acid fermentation of a polysaccharose should yield the same products as would their component hexoses. This does not always occur, and in some cases the fermentation of a disaccharose yields wholly different products from those produced in the fermentation of its constituent hexoses. This is particularly evident in the investigation of Grimbert upon the products of the fermentation of various carbohydrates by the same lactic



acid bacteria (See "Other Products"). In view of such exceptions, Kruse and H. Fischer believe that the lactic acid fermentation of a higher sugar does not always require a preceding hydrolysis. Their conclusions are perhaps open to the criticism that the hexoses themselves vary in their availability to lactic acid fermentation enzymes, as will be shown later.

In an early report, Bourquelot (1893) stated that maltose and sucrose were fermented directly in lactic acid fermentation. His work is, however, of great interest only from a historical standpoint.

Gayon and Dubourg present perhaps the best evidence that there are lactic acid bacteria which do not hydrolyze disaccharoses previous to their fermentation. In the fermentation of fructose by their cultures, mannitol was one of the products; when sucrose was fermented, no mannitol was formed if the acid products were kept neutralized. On the other hand, if sucrose was hydrolyzed to glucose and fructose, and the invert sugar presented to the organisms, mannitol was produced. From this line of evidence they concluded that these organisms fermented sucrose directly, and as such. They did not believe that the sucrose fermentation included the action of an endocellular lactase, as the fructose thus yielded should serve as a substrate for mannitol production. They also observed that in sucrose fermentations in which the acid products were not kept neutralized, mannitol was formed in the later periods of the culture. (Compare "Mannitol" under "Other Products") They believed that in these cases, the lactic and acetic acids which had accumulated in the system, hydrolyzed the sucrose; and that the mannitol had been derived by action upon the fructose thus produced. Gayon and Dubourg claim that maltose, lactose and also raffinose, are fermented in the same way by their cultures, - without previous hydrolysis to hexoses.

Recently, more conclusive evidence has been presented that disaccharoses are hydrolyzed by appropriate enzymes as a preliminary stage in their lactic acid fermentation by the more common lactic acid bacteria. Avery and Cullen (1920) have demonstrated the presence of endocellular invertase and other hydrolyzing enzymes in the case of the pneumococcus (which is itself

a lactic acid organism apparently closely related to the most common lactic organism of the dairy). As evidence of the fact that the production of lactic acid from disaccharoses is not due in this case to an immediate attack upon the 12-carbon sugar, it may be pointed out that the enzyme material used in their tests induced hydrolysis, but did not bring about the production of acid. The lactic acid zymase, if liberated, proved less stable to the conditions of their experiment, than did the hydrolyzing enzymes present in the enzyme mixture.

The possibilities of such differences in the stability of the members of enzyme complexes or enzyme mixtures, are suggestive of the difficulties to be encountered in investigations of intermediate products of microbial reactions.

The hydrolysis of disaccharoses into hexoses before their utilization by cells is supported by the work of E. Fischer and Lindner, and by the phenomena of general physiology, the following conclusion of Rogers, Clark and Davis seems well founded and furnished as definite a statement as can be made upon this question. In discussing the fermentation of sugars by lactic acid bacteria of the acid gas group, they state: "It is generally assumed that substances like sucrose must be hydrolyzed before constituent glucose or fructose can be utilized. While definite evidence of this is lacking we may assume it to be true."

Other conservative and reliable authorities concur with this conclusion that probably, at least in the case of most lactic acid bacteria, the particular hydrolyzing enzymes are present when disaccharoses undergo lactic acid fermentation. This, of course, does not preclude the possibility of exceptions in the case of some lactic acid bacteria.

b. Enzymes involved in "acid gas" fermentations.

The explanation of the character of the enzyme or enzymes involved in mixed lactic acid fermentations has been even more difficult. The gradual development of the interpretation of the nature of the enzymes involved in the acid gas type of lactic acid fermentation is evident in a summary of the work of Harden and Grey already reviewed.

Harden and Penfold, at the time they proposed the series of equations given under "Chemical Changes", assumed the presence of three enzymes, each responsible for a particular reaction yielding different products. Grey, in 1918, produced evidence in support of this theory, by means of showing a difference in the influence exerted by various factors upon the rates of the reactions of the different enzymes. At that time he reached the conclusion that the "enzymes of *B. coli* are, partly at least, independent of one another in their action; degradation of glucose is brought about by means of these independent enzymes acting either simultaneously or consecutively." His later papers, (1919, 1920), reviewed above, give such conclusive evidence of the independence of these enzymes that one is led to believe that the production of lactic acid in acid gas fermentation may well be the result of an enzyme not different from that involved in true lactic acid fermentation. The presence of enzymes yielding other products is apparently merely a characteristic of the species, rather than of the type of fermentation.

No evidence can be given to show that other enzymes are involved in the production of lactic acid in acid gas fermentation than those functioning in the production of that substance in "true" lactic acid fermentation.

#### c. Complications introduced by other factors.

The question of intermediate substances also complicates an analysis of the nature of the lactic acid producing enzymes, as it introduces a possibility of several enzymes being concerned, even in the "true" lactic acid fermentation of a hexose. The different optical forms of lactic acid produced present further questions concerning the unity of the lactic acid enzymes. (These will be discussed under "Stereochemical Lactic Acid Fermentation.")

In concluding a discussion of the enzyme or enzymes responsible for the production of lactic acid from carbohydrates, it is well to remember that, although lactic acid fermentation may be regarded as established as an enzymatic process, it is by no means certain by just what enzyme this is brought about, or whether more than one enzyme is concerned.

#### d. Physiological function of lactic acid bacteria zymase.

As stated before, in the discussion of the significance of the energy change brought about by the reaction induced by the lactic acid bacteria zymase, lactic acid fermentation is most important as a means of furnishing energy to the lactic acid bacteria. Hence, although the physiological function of perhaps most enzymes is exerted in the preparation of available food for the cell, the essential function of the lactic acid bacteria zymase is



the preparation or release of energy. It is not involved in the material nourishment of the cell, (its products do not serve as food), other than in the furnishing of energy for other life processes, some of which are endothermic.

## 2. Enzymes producing lactic acid from nitrogenous material.

Some lactic acid bacteria seem to possess an enzyme capable of converting certain nitrogenous substances into lactic acid.

In experiments of Kayser, (ref. Duclaux), in which he added peptone to certain sugar solutions, some of his lactic acid bacteria produced more lactic acid than could have been derived from the sugar alone. He believed that this increase in acid could not be explained otherwise than by the conversion into lactic acid of some of the nitrogenous material introduced in the form of peptone. Kayser has also observed production of lactic acid by lactic acid bacteria in 1% to 2% solutions of Chapoteaut peptone, which solutions gave positive Fehling tests neither before nor after boiling with hydrochloric acid. Koestler also reports lactic acid formation from peptone by his lactobacilli.

Some authorities explain the increase in lactic acid content of certain cheeses during ripening stage, after complete disappearance of lactose, by assuming the ability of lactic acid bacteria to produce lactic acid by action on casein.

The conversion of at least the simpler nitrogenous substances into lactic acid is not difficult to accept, in view of the easy conversion of some amino acids into lactic acid.\* (See "Substrate").

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\* Formation of lactic acid in animal tissue is apparently not dependent upon enzymes acting upon nitrogenous material. See following pages, under "Substrate".

### 3. Substrates of lactic acid producing enzymes.

#### a. General discussion, with table.

Lactose was the substrate of the first observed lactic acid fermentations, but it was soon found that many other substances underwent lactic acid fermentation. In the extensive investigations of W. Hennenberg upon the ability of many species of lactic acid bacteria to ferment different substances, it was found that all species investigated by him fermented glucose, fructose and galactose, most of them also lactose, maltose and sucrose; a few fermented pentoses, dextrin and starch, while many fermented polyvalent alcohols, as glycerol and mannitol. In the literature of lactic acid fermentation are reported other extensive investigations of the substances from which lactic acid is produced by lactic acid bacteria. A most extensive literature exists in regard to fermentation of different carbohydrates by the *E. coli* group. It belongs more properly to sanitary bacteriology than to agricultural lactic acid fermentation. See "Classification of Acid Gas Group".

Jensen (1919) furnished fermentation characteristics exhibited in many carbohydrates by 330 strains of "true" lactic acid bacteria.

Fred and associates report on the ability of their pentose fermenters to attack a variety of carbohydrates.

A complete review of this material is not pertinent to this discussion.



b. Other carbohydrates sometimes serving as substrates.

Other carbohydrates than those mentioned above may serve as substrates for lactic acid producing enzymes. Organic acids and their salts are fermented to lactic acid by certain bacteria.

Seifert and Rosenstiehl have both observed production of lactic acid by bacterial fermentation of malic acid. Fred, Peterson and Davenport report the ability of their pentose bacteria to convert malic acid into lactic acid.

c. Specificity of lactic acid bacteria enzymes.

It is seen that carbohydrates of diverse chemical constitution and configuration serve as substrates for the lactic acid producing enzymes of lactic acid bacteria. This renders rather difficult the strict application of the older theories of specific relation of enzyme to substrate. This phenomenon, however, is not in discord with more modern theories. The symase of the lactic acid bacteria exhibits Bayliss' "master key" relation to different substrates and furnishes an example of Beatty's "group specificity" ---but it does this to a much greater extent than do many other common enzymes.

Moreover, it is evident from the above table that the enzymes of different lactic acid bacteria differ in their ability to attack different substrates. Weigmann explains this from the standpoint of the lock and key theory of enzyme-substrate relation. He as-



sumes that different lactic acid bacteria produce enzymes of different stereochemical configuration, and that the ability of a species to produce lactic acid from a certain sugar depends upon its possession of an enzyme of spatial configuration, the image of that of the substrate. Such an assumption is conditioned, however, by the above modern conceptions of enzyme specificity.

d. Origin of lactic acid in animal tissue.

The occurrence of lactic acid in muscle tissue is well known. "The reaction of an inactive living muscle is alkaline, but upon the death of the muscle, or after the continued activity of the muscle, the reaction becomes acid, due to formation of lactic acid." It may be assumed with safety that this production of lactic acid is also an enzymatic reaction. The substance from which the lactic acid is formed must be the substrate of these enzymes. Different opinions are held regarding the substance from which lactic acid arises; some authorities claim that the muscle carbohydrates serve as the substrate, others, that protein substances furnish the substrate. "The strongest evidence favors a carbohydrate source." (Hawk).

The following investigators support carbohydrate origin:

- Spiro, 1877, Z. f. Physiol. Chem., I, III.  
Hoppe Seyler and co-workers, 1891-1894,  
Z. f. Physiol. Chem., XV, XVI, XVII,  
XIX.  
Lusk and Mandel, 1905, Amer. Jour., Physiol.,  
XVI, 129.  
Levene and Meyer, 1912, Jour. Biol. Chem., XI,  
361.  
Hawk, 1918, "Physiological Chemistry", 371.

The following investigators are the strongest supporters of protein origin. (Cited by Levene and Meyer).

- Minkowski, Arch. f. Exp. Path. u. Pharm.,  
1886, XXI, 67; 1893, XXXI, 214.  
Asher and Jackson, 1901, Z. f. Biol., XLI, 393.  
Neuberg and Langstein, 1903, Arch. f. Physiol.  
Suppl., 514.

#### IV. Enzymes of Other Metabolic Processes of Lactic Acid Bacteria.

##### 1. Nitrogenous substance hydrolyzing enzymes.

###### a. Rôle in the metabolism of lactic acid bacteria.

In lactic acid fermentation, the enzymes acting upon nitrogenous substances are important as a means of furnishing the lactic acid bacteria material for growth and for cell substance rather than in the direct production of lactic acid. Chemical analyses of lactic acid bacteria show that their cell substance is largely nitrogenous material. Moreover, Burton and Rettger and others claim that in media containing both proteins and sugars, (as is the case in the media of lactic acid fermentation), the sugar furnishes the required energy and the nitrogenous material furnishes the substance required

for growth and for the building of cells. Hence, it is best to consider these enzymes from the standpoint that their substrates are most important as a source of cell substance for growth and reproduction. It follows that, in many media, the ability of these enzymes to render the nitrogenous material available to the needs of the metabolism of the lactic acid bacteria will largely determine the physiological efficiency of all their life processes, including the lactic acid forming enzymatic processes discussed above.

b. Enzymes attacking derived proteins and peptides.

(1) Stimulation of "peptone" in medium.

It will be shown later that an increase in the "peptone" content of the sugar media in which lactic acid bacteria are growing usually results in

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\* In reporting the data of different investigators of the nitrogen metabolism of lactic acid bacteria, it is difficult to escape the use of the misleading term "peptones", which they have applied to the various nitrogenous substances contained in commercial peptone. This use of the word is objectionable here in a discussion of action of enzymes of lactic acid bacteria upon different nitrogenous substances, since it has been shown that commercial "peptones" contain a considerable amount of nitrogenous material of much simpler nature than peptones or proteoses. Moreover, Rettger and others have shown that apparently it is these simpler nitrogenous substances that are most easily utilized in bacterial metabolism. This must be considered in the interpretation of the availability of different nitrogenous materials to enzymes of lactic acid bacteria.

an increase in the amount of lactic acid formed. At least a large part of this stimulation of activity of the organisms is considered to be due to added food value of the medium. (The buffer value of the medium must also be considered, as will be shown later). This, then, in itself would require the presence of enzymes capable of attacking and utilizing the simpler nitrogenous substances.

(2) Demonstration of their action by measurement of their products.

Jensen (1904) showed that in sugar free peptone broth *Strep. lacticus* brought about cleavage of peptone with formation of ammonia and amino acids.

The introduction of ( $H^+$ ) measurements and of Sorensen's formal method of determining amino acids have produced evidence establishing the fact that common lactic acid bacteria bring about the formation of these simpler protein derivatives by the degradation of the above derived proteins (at least in low ( $H^+$ ) and in the absence of a fermentable sugar). Itano measured the production of amino acids in peptone broth. He found an increase in formal titrating nitrogen and a decrease in ( $H^+$ ) in cultures of *Strep. lacticus*. The difference in amounts of amino acids formed by this common lactic acid bacterium and by a virulent strain of *Strep. erysipaelatis* he attributes to influence of former habitat of these organisms upon their formation of proteolytic enzymes.

By the same method of measuring protein cleavage, Rosenthal and Patai demonstrated ability of other lactic acid bacteria to degrade such proteins. By means of ( $H^+$ ) measurements, Evans (1918) has demonstrated peptone degradation by *Strep. lacticus*.

From these results, enzymes attacking peptides, peptones, and possibly other derived protein material, may be assumed to be present and to be of significance to the common lactic acid bacteria, such as *Strep. lacticus*.



Further work in this field constitutes a part of the investigation to be reported in Part II of this work.

In all probability, the enzymes of many lactics show a selective preference for certain of the simpler peptide constituents of "peptone". It has been shown by Avery and Cullen that the proteolytic enzymes of pneumococci attack with greater avidity "peptones" of less complexity. (The American commercial peptones are further hydrolysed than the old Witte product.)

c. Enzymes hydrolyzing primary and conjugated proteins.

(1) Importance of their presence.

Although the presence of enzymes further hydrolyzing peptides, (at least under optimum conditions), may safely be assumed, the question of enzymes attacking higher proteins is a much disputed question. The presence of such enzymes in the lactic acid bacteria becomes very important in several agricultural lactic acid fermentations, especially in the ripening processes of cheese making. As casein is probably the most important protein in the media of agricultural lactic acid fermentations, the following discussion of these enzymes will be directed largely to the action of lactic acid bacteria upon this conjugated protein. That casein is a substance relatively resistant to bacterial attack is definitely established.

(2) Ability of different lactic acid bacteria to hydrolyze significant amounts of casein.

In such a diverse collection of micro-organisms as those included under the term of lactic acid bacteria, it is but natural that some of them possess enzymes capable of attacking the higher proteins such as casein.

In the fourth group are found many organisms which without doubt hydrolyze this protein. Most of these are the "acidoproteolytic cocci" of Gorini, (1904, 1912), or organisms such as *Staph. pyogenes* (many of which are often not considered as "true" lactic acid bacteria). These lactic acid bacteria seem to possess proteolytic enzymes of peptic character. (It will be shown in Part II of this paper that the proteolytic enzymes of some members of this group are very tolerant to high ( $H^+$ )'s). However, many of the so-called "liquefying" lactic acid bacteria, as *M. acidilactici liquefaciens*, are so named because of their action on gelatin rather than on casein (Kayser, 1915).

Besides members of the fourth group of lactic acid bacteria, many of the lactobacilli seem to possess enzymes capable of hydrolyzing casein. Hastings, Evans and Hart, Barthel (1913), Bertrand and co-workers, Finkelstein, and others, have observed utilization of the casein in milk cultures of the bulgaricus group.

In the case of many of the common lactic acid bacteria, especially the *Strep. lacticus* group, conflicting claims are made concerning their possession of enzymes hydrolyzing proteins like casein.

Some of these lactics appear to be indifferent to the presence of the higher proteins. In experiments with *Strep. lacticus*, Barthel (1913), Weigmann (1898), Schirokich, and Gorini, did not observe any appreciable proteolytic action upon casein. Freudenreich and Thöni (1904) reported much stronger proteolysis of casein by other strains of this group. Later, Barthel (1915) found that some strains of the common lactic bring about a significant degradation of casein if neutralizing substances are present in the medium. Barthel's (1919) latest investigation with a number of strains showed that the ability of most members of this group to attack casein in a neutral medium is greater than was formerly supposed. Von Freudenreich and co-workers found that, although some of his lactic acid bacteria were practically without action on casein, others were able to hydrolyze this protein with the formation of soluble nitrogenous compounds, provided that the lactic acid (produced from the lactose) was kept neutralized. Jensen (1904) obtained like results with the *lactobacillus*, *B. casei*.

### (3) Possibility of latent enzymes.

In support of similar results, Mase claims that many lactic acid bacteria possess the enzyme casease, but that this enzyme usually has only a slight action, because it is soon inhibited, after the milk reaches a certain ( $H^+$ ). Quite the same conclusion is reached by Weigmann (1910), who states that a degradation of casein or caseinogen occurs with most true lactic acid bacteria only if the acid produced is kept neutralized. Jensen makes a similar statement.

(4) Factors conditioning, and often inhibiting, proteolytic enzymes.

(a) ( $H^+$ )

From this standpoint, even the common lactic may possess protein hydrolyzing enzymes which, however, in many cases are inhibited in their casein attack by the ( $H^+$ ) resulting from the production of lactic acid by the action of the zymase upon the sugar in the medium. This inhibitory influence of ( $H^+$ ) upon the proteolytic enzymes of lactic acid bacteria is in accordance with established ( $H^+$ ) zones which limit the activity of enzymes. Upon the basis of the above assumption, Gorini (1915) goes so far as to separate his "acidoproteolytic" lactics, which are able to attack casein in acid combination, from the more common "alkalinoproteolytic" lactics, which he assumes can attack casein only when in alkaline, or at least neutral, systems.

Besides this direct action of ( $H^+$ ) upon the enzymes itself, it is possible that in high ( $H^+$ ) changes in the condition of the protein substrate render it less available to the particular enzymes. Mase believed that the change of the caseinogen from its former colloidal solution state made the casein less susceptible to the action of the assumed casease enzyme. This is of doubtful import.



(b) Influence of presence of fermentable sugar upon proteolytic enzymes.

The presence or absence of a fermentable sugar may determine whether or not active proteolytic enzymes are secreted by the lactic acid bacteria. This may be considered due either to a protection afforded the protein through the selective action of the bacteria for the sugar, or, as suggested above, to the ( $H^+$ ) which results from the production of lactic acid from the sugars when present, and which prohibits the functioning of proteolytic enzymes.

Many investigations of the influence of the presence of fermentable carbohydrates upon proteolytic enzymes are reported in the literature.

Kendall is one of the staunchest supporters of the sparing action of sugar upon the protein metabolism of bacteria. Effront states that fermentable carbohydrates exert a profound influence upon the proteolytic enzymes of lactic acid bacteria. Although many lactobacilli possess quite limited proteolytic activity in milk and other sugar media, Effront believes that these lactic acid bacteria, if present in a nitrogenous medium in which sugar is absent, will act in a different manner. "The ferment here finds no more sugar, and so it produces no lactic acid; but its proteolytic activity, which formerly was entirely latent, is now, on the contrary, accelerated. The bacterium, which was at first a very excellent ferment for carbohydrates, has become a ferment for nitrogenous materials; it secretes tryptases and amidases abundantly and thoroughly transforms the residues from albuminoid foods."

However, the present tendency is to explain the greater part of the influence of the presence of fermentable carbohydrate upon proteolytic enzymes upon the basis of ( $H^+$ ) rather than upon a sparing action of the sugar itself.

Berman and Rettger state that "the ( $H^+$ ) plays the important rôle in the inhibition of nitrogen metabolism in a medium containing a fermentable sugar. The failure of certain organisms to attack proteins in the presence of carbohydrates . . . is dependent on a coincident rise in the acidity of the medium."

Significant production of enzymes attacking higher proteins is believed to be delayed and, with most micro-organisms, to occur only after metabolism of more readily utilisable substances has afforded considerable growth. With certain micro-organisms, apparently with common lactics, the metabolism of the more available substances, (it is established that a fermentable sugar is most available to lactic acid bacteria), may result in environmental conditions inhibiting the micro-organism before protein attacking enzymes are produced.

Upon this basis may be offered a dynamic explanation of the influence of carbohydrates upon protein metabolism; the difference in ability of different micro-organisms to attack protein in the presence of fermentable carbohydrates depends upon the relative speed of formation and action of the enzymes attacking these substances. In case of many lactic acid bacteria, where the equilibrium point usually existing is to the disadvantage of protein metabolism, it may be displaced by changes in the environment system. This is manifested by

the functioning of proteolytic enzymes of many common lactics in a heavily buffered medium, even in the presence of a fermentable sugar.

(5) Evidence from applied lactic acid fermentations.

(a) Microbial association.

The question of an enzyme attacking casein or caseinogen is again of interest in a study of the microbial association in lactic acid fermentation of milk. Although this will be discussed later, it may be stated here that many lactic acid bacteria seem to be limited in their growth in milk by the content of simpler nitrogenous material, and to be dependent upon the enzymes of associate micro-organisms for an adequate supply of this part of their food. This seems to offer further evidence that if these lactic acid bacteria do possess an enzyme capable of hydrolyzing the protein casein, it is not active under the usual ( $H^+$ ) conditions prevailing during lactic acid fermentation of milk.

Further suggestions on the ability of lactic acid bacteria to utilize casein is given in the following discussion of the rôle of their enzymes in the curing of cheese.

(b) Cheese ripening.

The ability of lactics to hydrolyze casein is an important question in the ripening of cheese, but the exact significance of enzymes of lactic acid bacteria in the changes which occur in casein during the curing of cheese is not definitely established.

Certainly it is different in the various types of cheeses and depends upon many factors introduced by the different making and curing processes to which the coagulated casein is subjected.

The rôle of casein attacking enzymes of lactic acid bacteria in cheese ripening must vary with the conditions given; its interpretation is difficult and the reports of many investigators are at variance.

Freudenreich and Jensen, much of whose work has been concerned with Emmenthal cheese, have ascribed great importance in cheese ripening to action upon casein by enzymes of lactic acid bacteria, especially of lactobacilli. Orla Jensen (ref. Barthel, 1915b) sums up their position on this question as follows: "It is the lactic acid bacteria, and among these, at least in Emmenthal cheese, especially the lactobacilli, which play the principal rôle in the decomposition of the casein." This he claims is brought about by an endoenzyme freed from the cells of lactobacilli by autolysis.

Hastings and associates suggest presence of casein attacking enzymes by this group. They showed that lactobacilli multiply in Cheddar cheese after the lactose has been consumed (largely by the lactic streptococci). "Since they (lactobacilli) develop after the fermentation of the milk sugar, they must have some other source of carbon and of energy than milk sugar", and casein hydrolyzing enzymes are probably functioning in the preparation of casein for that purpose.

Barthel, (1915b), accepts the importance assigned by Jensen to the casease of lactobacilli but, in addition, would attribute a significant rôle to similar enzymes of *Strep. lacticus*. "In view of the fact that in many hard cheeses, *Strep. lacticus* predominate, at least in the first few months and since the cheese is usually held at 15-20° C., it seems that they should be assigned more importance in the ripening process than is usually given them." Jensen (1919) agrees with Barthel.

The American investigators of Cheddar cheese, (Hastings, Evans and Hart (1912), Hart, Hastings, Flint and Evans (1914) ), while "certain that the (*Strep. lacticus*) *Pact. lactic acid* group is an essential factor in the ripening", seem to attribute these lactics' rôle



chiefly to indirect action of the lactic acid produced by the lactic acid zymase. The rôle of lactic acid bacteria in the ripening of such cheeses, in which considerable lactic acid fermentation takes place during the making process, is not limited to the action of their casein hydrolyzing enzymes. A great part of the change in the condition of the nitrogenous constituents of cheese is undoubtedly due to the action of the lactic acid as an activator of the pepsin of the rennet extract and other enzymes of the milk. Probably also the lactic acid serves as a means of bringing about certain changes in the condition of casein by direct action of the acid itself. (Van Slyke and associates, 1905, 1907; Bosworth, 1907).

Gorini's work on the acid lab group seems to establish their possession of casein hydrolyzing enzymes. Such an enzyme is suggested by the fact that these lactics also possess a casein coagulase. Their rôle is probably limited to Parmesan, Grana and similar long cured hard cheeses.

(6) Conclusion as to the general ability of lactic acid bacteria to hydrolyze casein.

It is seen that all of the chemical changes undergone by the casein during the curing of cheese are not due to casein hydrolyzing enzymes of lactic acid bacteria, and that investigations of these phenomena have not established definite proof of the presence and active functioning of such enzymes in all lactic acid bacteria. However, it is perhaps possible to sum up the evidence on this question as follows:

Many of the lactobacilli possess enzymes capable of attacking casein; many strains of the *Strep. lacticus* group also possess such enzymes, usually less active than those of lactobacilli. Not only the functioning, but probably also the elaboration of these enzymes of both groups are usually conditioned, at

least in degree, by the presence of sugar in the medium, by temperature, and especially by the ( $H^+$ ) of the system. The "acid lab" group of Gorini possess casein hydrolyzing enzymes which, apparently, are not so dependent upon a low ( $H^+$ ).

## 2. Protein coagulating enzymes.

### a. Variation among the lactic acid bacteria.

The question of the possession of protein coagulating enzymes is still more undecided than that of protein hydrolyzing enzymes. It is probable that here, too, the different lactic acid bacteria differ --- that some lactic acid bacteria possess such enzymes to an extent easily demonstrated; that others possess them to a less, and not easily demonstrated, extent; and that still others do not possess them at all. The following evidence has been presented concerning their presence in the different groups of lactic acid bacteria.

### b. Reports on lab enzymes of the different groups.

Within the fourth group are included bacteria which Gorini claims produce a caseinogen coagulating enzyme as well as enzymes producing lactic acid fermentation. The possession of such enzymes has recently been demonstrated in a study of several strains of *Staph. pyogenes*, which organism is usually considered the type species of that group. (Barnes 1921).

With the other groups of lactic acid bacteria, such definite demonstrations have not been made.

Ducleux thinks it possible that at least some lactic acid bacteria produce a coagulating enzyme. Many authorities assume that in case of the typical lactic acid bacteria, coagulation of the caseinogen is induced surely by the ( $H^+$ ) incident to the production of lactic acid. Rogers and Davis, however, claim that certain lactic acid bacteria coagulate milk at a lower acidity than would account in itself for the production of a curd. Avery and White found that the addition of neutralizing substances in excess of the acid produced failed to prevent coagulation of milk by their lactobacilli. Finkelstein and Penrose also report like results with other lactobacilli. These results can be interpreted in favor of the presence of a caseinogen coagulating enzyme.

On the other hand, with the common lactic acid bacteria, Schirokich, Gorini (1904), and Jensen (1904) claim that *Strep. lacticus* produces no lab ferment. Bennecke suggests that, in view of the very doubtful existence of a casein digesting enzyme in the case of the more common typical lactic acid bacteria, it is also very doubtful if a caseinogen coagulating enzyme is produced. (He bases his assumption upon the general rule that most bacteria producing a rennet-like coagulation of milk usually later digest the curd.) His conjecture is weakened, however, by the proof by Barthel and others of casein hydrolysis by the common lactics.

#### e. General conclusions as to lab enzymes of the lactic acid bacteria.

It is evident from the above that much the same conclusion must be drawn in regard to the presence of protein coagulases as was the case with protein hydrolyzing enzymes. The fourth group of lactic acid bacteria have been shown to produce protein coagulating enzymes. The lactobacilli also seem to include organisms which seem to possess such enzymes,

but to a less evident extent. The *Strep. lacticus* group, if indeed they possess them at all, have not been shown to possess active or easily demonstrated protein coagulating enzymes.

### 3. Other enzymes of the lactic acid bacteria.

The lactic acid bacteria also possess other enzymes which will not be discussed here, although in many cases their action is very important.

Among them, may be mentioned the "reductases" which are said to bring about the reduction of dyes to their leucobases.

It must be admitted, however, that definite demonstration is still lacking that such changes are always of purely enzymatic nature (as we now understand the term "enzymatic".) There seem to be many factors involved in reduction, which at present are impossible to explain upon a simple basis. Many of these same factors may also be concerned most intimately, in the reaction of lactic acid fermentation itself. In this connection, certain recent work of Harden is of some significance, particularly since in a study of the mechanism of lactic acid fermentation, it is important to recognize the possible meaning of other, reducing reactions that may occur in the same system.

He believes that the reduction of dyes is effected by enzymes, but that the operation of their actual reducing action is conditioned by the presence of certain substances. (Harden and Silva (1915)).



Harden and Norris (1915) found that suspensions of washed dried yeast failed to reduce methylene blue. Upon addition of free lactic acid and of sodium lactate, such suspensions reduced the dye. (The lactic acid itself seemed to be oxidized in the reaction: a part of it, at least, to acetaldehyde.) Washed rabbit muscle was also found to lose its power of reducing methylene blue. This power was restored by various substances, which, however, were different than those effective in the case of yeast. The authors believed this suggested different enzymes.

The more modern investigations of recent years upon the reduction and oxidation reactions involved in other physiological processes, have not yet been extended to the lactic acid bacteria. Wieland has studied the oxidation process induced by suspensions of acetic bacteria. The same investigator has made a valuable study of the accelerating agent involved in the "reducing" and other reactions of raw milk. Dakin (1921) believes that Wieland's results show that the oxidase, reductase and aldehyde-mutase effects of milk are due to one and the same ferment, a "dehydrase". Wieland, as well as a number of other investigators, have shown a striking similarity between the "reductions" induced by ferments and those activated by inorganic catalysts.

In his review of the recent extension of our knowledge of physiological oxidations and reductions, Dakin emphasizes the importance to the future interpretation of these processes. "It would therefore appear very probable that many other long cherished oxidase and reductase reactions of living cells will have to be reviewed and that the effects which have hitherto been ascribed to them will be found to represent one or other phase of a series of concurrent reactions primarily induced by a 'dehydrase'." The consequences of such a revision can hardly be estimated at the moment, but that it will upset many long established preconceptions is certain."

Jensen (1919) also claims that "true" lactic acid bacteria do not possess enzymes for the reduction of nitrates, which are a common property of members of the acid gas group and also of many of the fourth group of lactic acid bacteria.

The absence of catalase, which is a quite generally distributed enzyme among bacteria, is suggested by Beierjenk and by Jensen (1919) as a characteristic of "true" lactic acid bacteria. (It has been shown, however, that the butyric acid bacteria also quite frequently lack this enzyme.)

Many of the lactic acid bacteria also possess enzymes capable of attacking the salts of organic acids (see references under "Reversal of Reaction"); those producing gas are assumed to possess enzymes attacking formic acid and formates, while others seem to possess enzymes attacking lactates (Jensen 1904).

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5.

THEORY OF THE DIFFERENTIAL CALCULUS.

A. DIFFERENTIAL CALCULUS.

B. DIFFERENTIAL CALCULUS.

C. DIFFERENTIAL CALCULUS.

# INFLUENCE OF THE ENVIRONMENT UPON LACTIC ACID BACTERIA

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## INFLUENCE OF THE ENVIRONMENT UPON LACTIC ACID BACTERIA

### A.. PHYSICAL INFLUENCES

#### I. Heat.

##### 1. Temperatures within range of growth.

##### a. General discussion of "cardinal points".

The temperature relations of micro-organisms are usually considered upon the basis of three so-called "cardinal points";-- maximum, optimum, and minimum temperatures for the growth of particular organisms. The difficulty of determining these points with any degree of accuracy is much greater than might be supposed. In a review of the results obtained by different investigators working with supposedly the same lactic acid bacteria, flagrant discrepancies are evident in reports on all of these cardinal points.

Tables showing the optimum, minimum and maximum temperatures as reported in the investigations of a number of species of lactic acid bacteria are given by Weigmann (1910) and by Hennenberg. The table by Weigmann, at least, is of little value as it includes reports of the early authorities, some of which are decidedly at variance with the present idea of the temperature relations of the same types of lactic acid bacteria.

Probably nothing more should be presented than a short review of the temperature relations exhibited by the four groups of lactic acid bacteria, which has been given in greater detail in the preceding division (see "Lactic Acid Bacteria").

b. Temperature relations of the groups of lactic acid bacteria.

It may be repeated here that the members of the *Strep. lacticus* group are able to grow fairly well at 10° C.; their optimum temperature seems to be around 32° C.; at temperatures above 43° C. but few strains are able to grow. The members of the acid gas group possess higher temperature relations, with their optimum at 37° C. and with minimum and maximum temperatures also somewhat higher. The lactobacilli exhibit the highest optimum temperature, with many strains around 43° C.. The members of the fourth group include organisms showing a wide zone of temperatures within their range of growth. Many of them grow well at very low temperatures, although their type species possesses an optimum temperature of 37° C..

c. Bases of determinations of optimum temperature.

Conflicting results reported for temperature relations of the lactic acid bacteria are probably due largely to methods employed in their investigation. Determination of the optimum temperature for the lactics should perhaps best be done by counting the cells. Many results are untenable because the investigators did not eliminate other factors. Determination of optimum temperature upon basis of time for coagulation of milk is misleading, as this phenomenon is influenced by temperature as well as by acid production; the possibility of presence of lab enzyme

or casease introduces other sources of error.

Such of those methods as are based upon the measurement of products of the micro-organism and its enzymes are probably fitted only for determinations of the optimum temperature for the production of a particular product. This is particularly true in cases where products are measured at a late stage of the growth of the culture, as here it is difficult to separate those products arising from purely enzymatic action from those produced by the life activity of the growing cells. (Compare Jensen, 1919).

d. Importance of knowledge of temperature relations.

The importance of a knowledge of the optimum temperature of the lactic acid bacteria cannot be overestimated. When present in mixed culture in the natural media of agricultural lactic acid fermentations, the temperature may be the factor determining what group of the lactic acid bacteria will predominate, or even whether lactic acid fermentation will take place at all. This relation is especially evident in the natural fermentation of milk.\*

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\* Compare Beierjink, 1904, K. J., XV, 262.



In pure culture lactic acid fermentations a knowledge of optimum temperature is necessary in order to control the fermentation so as to yield best results.

Although the preceding discussion has had particular reference to optimum temperatures, a knowledge of minimum and maximum temperature relations becomes of importance in many cases. Within the range between minimum and optimum, temperature exerts its influence chiefly by determining the rate of growth of the lactic acid bacteria. At temperatures considerably below the optimum, the life processes may be suspended, but often they proceed at a very low velocity, as shown under "Effect of Low Temperature."

Jensen (1919) shows that the minimum and maximum temperature of lactic acid bacteria may vary with the medium used. Results of investigations to be reported in Part II of this paper show that the maximum temperature may also vary with the size of the inoculum.

e. Other factors to be considered.

The influence of temperature upon lactics is usually considered almost wholly from the standpoint of its effect upon the rate of growth. It seems, however, that the rôle of temperature in the life of the lactic acid bacteria is a more extended one. Many investigators have found that temperature of growth of the lactics is of considerable moment in determining the direction, as well as the rate, of their life processes.

Corini (1912) reports that the casein destroying powers of his lactics are favored by low temperatures, while their lactose fermenting powers are greater at higher temperatures. A similar relation between low temperatures and casein proteolysis by lactic streptococci is reported by Barthel. Another example of the influence of temperature upon the direction of lactic metabolism has been observed in the "ripening" of cream for butter. Here it is reported\* that temperature of incubation is an important factor in determining the production of those products of lactic acid fermentation which contribute the odor and flavor to the fermentation mixture.

## 2. Influence of high temperatures upon lactic acid bacteria.

### a. Interpretation of "thermal death point".

The usual basis upon which the heat resistance of micro-organisms is considered is the rate of death, rather than the older so-called "thermal death point" determinations. This is a more reasonable basis, as it recognizes the course and nature of the disinfection process, (in this case, probably disinfection by hot water). Moreover, in many cases, especially in agricultural lactic acid fermentation, it is more important to know what temperatures for a certain period of time are required to kill the majority of cells, than the temperature required for complete sterilization. This conception is now recognized by most authorities and has been emphasized

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\* Marshall --- personal communication.

recently by Jensen (1919) in relation to a study of lactic acid bacteria.

There is, however, a more or less definite relation, at least a resemblance, between the results obtained by studies from the two standpoints, provided the conditions of the experiment are the same. The results reported in the following pages, especially those of the early workers, should be considered with the above statements kept in mind, as most of the early work has been done from the "thermal death point" standpoint.

b. Factors influencing "thermal death point" determinations.

Discordant results are reported in the literature concerning the "thermal death point" of lactic acid bacteria. A large part of this discordance is due to the different methods employed in making these determinations, to the influence exerted by the medium in which the bacteria are suspended during the heating, and to the differences in resistance offered by cells of different ages and in different states of vitality. Many other factors are also involved, whose influence is evident in the results reported in determinations of thermal death points of all bacteria. Furthermore, in the heterogeneous group of lactic acid bacteria there naturally exists a large number of species differing widely in their

heat resistance; great differences exist also between strains within the species.

c. General discussion, with table.

In the interpretation of the results of investigations of thermal death points of the lactic acid bacteria, many of which give quite different results, the factors mentioned above must be kept in mind. The following table is from a compilation by Weigmann (1910).

TEMPERATURE (CENTIGRADE)		55°	60°	65°	70°	90°	100°
ORGANISM	AUTHORITY	TIME (IN MINUTES)					
Bact. aerogenes	Kayser	15'	5'	5'	5'		
Bact. lactis acidi	{Günther and	10'					
	{Thierfelder						
	{Schwitser	15'					
Bact. casei a	Freudenreich and Thöni	over 30'	over 5'	over 5'	short time	at once	
Bact. pabuli acidi	Weiss	over 60'	15'				

A compiled table, such as the above, is of relative value only, as it is limited to so great an extent by the different experimental conditions under which the results were obtained. Although the results are quite variable, it is evident that most lactic acid bacteria, (no typical forms of which are spore bearers), are not very resistant to heat. It is certain, however, that Kayser's (1915) general statement that "at least most species of lactic acid



bacteria are killed by exposure for a short time to a temperature of 65°-70° C.", is conditioned by factors to be discussed later.

d. Lactic acid bacteria showing high resistance to heat.

That some lactic acid bacteria possess comparatively high thermal death points for non spore-formers is evident from the results of many investigators.

Weiss and Dotterer and Breed have found some lactobacilli to possess rather surprising resistance to high temperatures. Rahe has found members of this group that in twenty-four hour broth culture survived exposure to moist heat at 65° C. for one hour. Similar results have probably been the basis for Jensen's (1919) generality --- "As a rule, those lactic acid bacteria which grow at the highest temperature can also stand the highest degree of heating." As might be expected, he found at least one exception.

Although as a whole, the other groups are not so resistant to heat as the lactobacilli, it will be shown below that some members of the other groups also exhibit higher heat resistance than would conform to Kayser's general statement.

e. Thermal death rate of lactic acid bacteria in milk.

(1) Greater resistance apparently exhibited in milk.

Much work has been done on this question, especially with the *Strep. lacticus* and the *B. aerogenes* groups, as many investigators, (Marshall 1897, Ayers and Johnson(1913, 1914, 1915), Rogers, and others),

found that some members of these supposedly heat susceptible groups survived the temperatures employed in the pasteurization process. The results of much of this work seem to indicate that, when suspended in milk, higher temperatures are required to kill the lactic acid bacteria than in the case for broth suspensions, used in the usual determination of "thermal death points". (Brown and Peiser)

It has been shown in this laboratory that strains of *B. coli* and of *Strep. lacticus* were killed within five to eight minutes at 62.8° C., if suspended in neutral broth or physiological salt solution. The same strains, when suspended in milk or cream, were able to survive thirty minutes at the same temperature.

## (2) Factors involved.

In milk as a medium, the factors determining the lethal effect of heat on bacteria are especially evident.

Among the factors entering into this protective action of milk, may be mentioned the following: the surface pellicle forming on raw milk during exposure to air, the protective action of which is due partly to lower temperature at the surface and partly to the nature of the membrane itself (Russell and Hastings); a protective action of the casein or albumin present, due possibly to a film formation around the cell (Brown and Peiser, Rosengren); presence of fat itself, especially in cream, may have a direct protective action (Rosengren), and also a low percentage of water would give bacteria present the advantage of the lower sterilizing efficiency of dry heat as compared to moist heat (Brown and Peiser). Probably the pasteurization process itself introduces other factors which, added to the apparent protective influence of milk, offer further protection to milk bacteria.

Whether due to unusual heat resistance of a few lactic individuals, or to some protective action

of milk itself, or to methods employed in pasteurization, it is known that many strains of lactic acid bacteria (some of which may succumb, if in broth, to temperatures several degrees below that of pasteurization) are able to withstand temperatures of 60°-65° C. for 30 minutes under the conditions existing in the commercial pasteurization process.

f. Significance of thermal death relations exhibited by lactic acid bacteria in the pasteurization process.

(1) Microbial balance.

The "thermal death point" of lactic acid bacteria when suspended in milk is of significance from several aspects in the pasteurization of that food substance. Its most important aspect is the influence of that process upon the delicate equilibrium existing between the different types of microorganisms in milk. It is this balance which determines the type of alteration that will be induced in the medium.

The growth of lactics brings about a harmless and the most desirable, as well as the most evident, alteration of milk; moreover, it serves to inhibit other less evident, but undesirable and even dangerous, changes in the milk. Under influence of this belief, the early authorities suggested that the consequent shift of the microbial equilibrium to the

disadvantage of lactic acid fermentation would expose pasteurized milk to other changes more undesirable and difficultly recognized. However, this objection to pasteurization is not in keeping with later research, as it is founded upon underestimation of the heat resistance of many strains of lactics or upon experiments using higher temperatures than the one now usually employed in the processing of milk.

Marshall, in 1897, had shown that some lactics survive the process. Rogers, in 1905, Maze, in 1907, Ayers and Johnson, in 1910, and others, also reported the survival of some lactic acid bacteria. Later investigations have shown that the lactic acid bacteria not only are not eliminated from the milk, but "that the percentage of the acid group is increased by pasteurization at low temperatures, while the other groups (inert, alkali forming, and peptonizing) are decreased in their percentage of the total flora."

The following figure taken from Ayers and Johnson's (1913) results shows that the temperature employed is a potent factor in determining the moment of the different groups in the microbial balance extant in the processed milk.



RAW  
MILK

PROCESSED MILK  
MILK PASTEURIZED FOR 30' AT

62.8°C

71.7°C

76.7°C

82.2°C

87.8°C

93.3°C

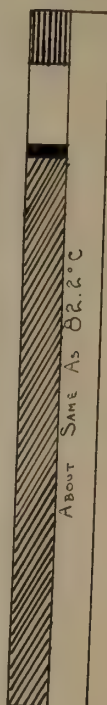
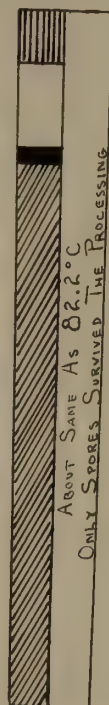
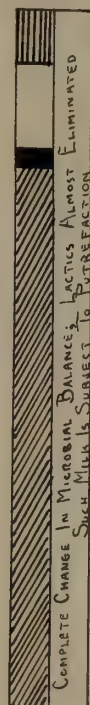
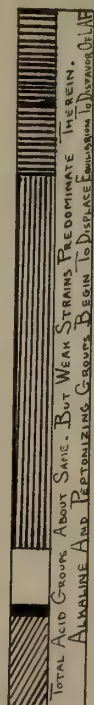
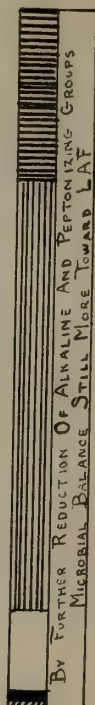
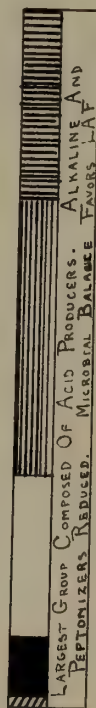
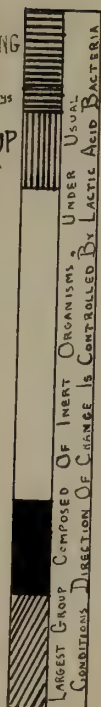
ACID  
COAGULATING  
GROUP  
coagulate milk  
less than 14 days

ACID GROUP  
do not coagulate milk  
within 14 days

INERT  
GROUP  
litmus milk

ALKALI  
GROUP  
litmus milk

PEPTONIZING  
GROUP  
milk



INFLUENCE OF TEMPERATURE OF PROCESSING UPON MICROBIAL BALANCE  
EXTANT IN PASTEURIZED MILK

FROM: AYERS AND JOHNSON, 1913, B.A.I., BULL 161., p 62.

The preceding figure shows only percentage relations between the groups; naturally, the absolute number of lactics is greatly reduced. However, as seen from the graph, pasteurized milk contains a sufficiently large percentage of lactic bacteria to ensure the lactic acid fermentation of the product and a consequent check on putrefaction, provided that the heating process is at temperatures near 62.8° C.. With high temperatures, the earlier objection becomes valid.

By the assurance that the lactics possess control of the direction of change finally undergone by pasteurized milk, the only sanitary objection to pasteurization has been overcome. It is now conceded that commercial pasteurized milk will undergo the usual lactic acid fermentation, because of the survival of lactics, although the fermentation is delayed, because of decrease in absolute numbers of lactic acid bacteria in the original germ content of the milk.

Weigmann and associates (1916) have also investigated the influence of pasteurization, upon the microbial balance of different samples of milk.

They found, as had the American workers, that in almost all cases the relative number of lactic bacteria was increased by pasteurization. Their report includes studies of the changes in value of the ratio between lactic acid bacteria and other bacteria and other bacteria in pasteurized milk, when held for certain periods at different temperatures. They have also attempted to establish ratios between the "strong" and the "weak" lactic acid bacteria, to the non-lactic types present. (Their differentiation between "strong" and "weak" lactics, (by means of the size of the clear zones produced on milk-agar plates) might prove rather difficult.) Their work does, however, raise the question of whether certain strains of lactics which may take a prominent part in the souring of raw milk, are replaced by other more heat resistant strains in the souring of pasteurized milk.

## (2) Pathogenic streptococci.

Following the above discussion of the heat resistance of lactic streptococci when suspended in milk, as in the pasteurization process, it is pertinent to consider the heat resistance of pathogenic streptococci which are often present with them in the flora of raw milk. The discussion of "Groups of Lactic Acid Bacteria" showed the close relationship of pathogenic and lactic streptococci as evidenced by the exceedingly close similarity in morphological and cultural characters. Fortunately, a greater difference exists in their ability to withstand the effect of temperatures near  $62.8^{\circ}\text{C}$ . for

30 minutes in milk.

While many lactic streptococci survive this heat effect, their pathogenic relatives have been shown to be unable to do so in the investigations by workers in the Dairy Division. In a recent paper, Ayars, Johnson and Davis (1918) summarize the results of investigation of this question: "Experience with the use of properly pasteurized milk and determinations of the thermal death point of pathogenic streptococci by various investigators indicate very clearly that the thermal death point of these organisms is relatively low and that they are rapidly destroyed by proper pasteurization." This, of course, does not preclude the presence of pathogenic streptococci in pasteurized milk by reason of contamination subsequent to the heating process.

Balter (1921) has compared the thermal death rate of lactic streptococci from human lesions with strains from milk. He found that, in some large inocula were introduced, a few cells of the pathogenic strains might survive 30 minutes' heating at 60° C. in



milk. (It is improbable, however, that such large numbers of heat-resistant pathogenic streptococci would be present in milk before the pasteurization process as were present at the beginning of Salter's tests.)

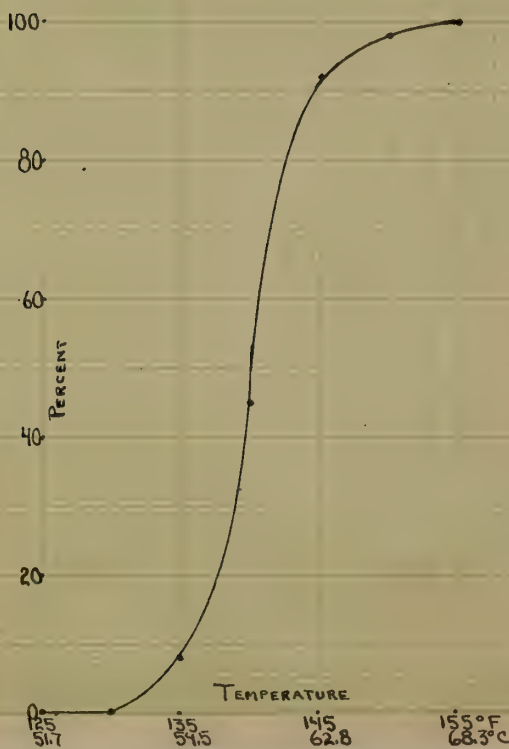
### (3) *B. coli* index.

The question of ability of members of our first group of lactic acid bacteria to survive temperature of 62.8° C. for 30 minutes, in milk, is also of significance in pasteurization. By many, who assumed that all strains of *B. coli* and its closer relatives were killed by this treatment, tests for the presence of these organisms in pasteurized milk are used as a criterion of the efficiency of the process.

Many investigators, (Rosenbren, Gage and Stoughton, De Jong and De Graff), however, have shown that some strains of *B. coli* are more resistant. Below, are given the results of an extensive investigation by Ayers and Johnson (1910) on the heat resistance of many strains of the colon organism when held in milk at different temperatures for 30 minutes.

HEAT RESISTANCE OF A NUMBER OF STRAINS OF  
B. COLI

PERCENT OF CULTURES KILLED AFTER 30' HEATING  
IN MILK AT DIFFERENT TEMPERATURES



As shown by the inflection point in the curve, 62.8° C. for 30 minutes is a critical heating period for many strains of the colon bacillus. Further, their results show that the occasional survival of certain strains is usually due to particular heat resistance of a few cells rather than to a high majority thermal death point.

Their conclusion, based on the above findings, may be taken as a statement of the present status of this question. "The colon test as an index of the efficiency of the process of pasteurization is complicated by the ability of certain strains to survive a temperature of 145° F. for 30 minutes and to develop rapidly when the pasteurized milk is held under certain temperature conditions which might be met during storage and delivery. Consequently the presence of a few colon bacilli in pasteurized milk does not necessarily indicate that the milk was not properly heated. The presence of a large number of colon bacilli immediately after the heating process may, however, indicate improper treatment of the milk."

#### g. General significance of heat resistance of lactic acid bacteria.

The "thermal death points" of lactic acid bacteria are important from two standpoints. In many cases it is desirable to prohibit lactic acid fermentation of a substance in which lactic acid bacteria are present, as in the media of alcoholic fermentation, in whey and skimmed milk to be used for stock food or in other food substances. Here, a knowledge of heat

resistance of the lactic acid bacteria is necessary in order to eliminate the lactics. In other cases, where lactic acid fermentation is desired, it is necessary to avoid temperatures which will exert a lethal effect upon lactic organisms.

### 3. Relation of lactic acid bacteria to low temperatures.

#### a. Retardation of life processes.

The immediate effect of low temperatures is not so marked as that of high temperatures. Many times lactic acid bacteria are apparently indifferent to considerably below their optimum temperature. In such cases the general effect of these temperatures is a decreased velocity and even suspension or inhibition of all life processes.

Many results of ice cream investigations reveal no appreciable increase or decrease in the number of lactics during cold storage of this product. The comparative inhibition effect of low temperatures is seen in results of Rahn and associates (1908), who found the lactic acid bacteria developing on plates held at 4° to 5° C. about one third of the number developed at the optimum temperature.

Life processes of lactics may proceed under low temperature conditions, but at a very low velocity.

Fennington (1908) found some acid formers growing slowly in milk held at 0° C.. In Brown's (1912) experiments on butter held at -3° F. to +3° F., production of lactic acid occurred at expense of lactose, but he does not state whether the lactic acid was due to action of living lactics or liberated enzymes. Luxwolda (1911) furnishes many more examples of growth of lactics at low temperatures, together with many references to the literature of investigations of influence of low temperature upon lactic acid bacteria.

b. Occasional germicidal effect.

The possible effect of low temperature extends from the above comparative retardation and inhibition to a definite lethal action. This is usually evidenced as a slow and more or less orderly progressive decrease in numbers, as in the case of drying. Under usual conditions, this decrease in numbers does not reach to complete disinfection of the medium.

MacFayden and Rowland found *B. acidilactici* and *E. coli* survived 10 hours' exposure to  $-252^{\circ}\text{C}.$  Rogers found no serious loss of vitality of *Strep. lacticus* upon exposure to  $0^{\circ}\text{C}.$

However, quantitative germicidal effects are revealed by many investigations. This effect of low temperature is conditioned by certain factors. Prolonged suspension of metabolism may result in the death of many cells, when lactics are held under low temperatures in certain media. The effect of degree of cold itself does not seem to be of much influence; apparently there is no rapid acceleration of the death rate following depressions of temperature below the freezing point comparable to that which gives rise to the so-called "thermal death point" in disinfection by high temperatures.

The actual freezing of the medium and the resulting mechanical influences of solidification and crystallization in the medium seem to be the most important lethal factors.



Rahn (1908) has shown that the freezing point of the liquid in salted butter is depressed, (by its salt content), considerably below the usual cold storage temperatures. He and his associates found that this was accompanied by a decrease in the death rate of the lactic acid bacteria in such systems. While above the freezing point, the death rate of lactics runs parallel in salted and unsalted butter, at temperatures below freezing the rate of death was greater in unsalted samples. Later work on *B. coli* by Keith and by Hilliard and Davis offers further evidence of influence of the actual freezing of the medium. Intermittent freezing is known to accelerate the death rate of bacteria.

The medium in which the bacteria are suspended is also an important factor.

Keith has shown that the death rate of *B. coli*, when frozen in diluted milk, increases with the dilution and that glycerin solutions offer still greater protection. Hilliard and Davis have also shown that milk and cream offer protection to bacteria subjected to low temperatures, and believe this protection to be due to the colloidal and solid matter in suspension in this medium.

#### c. Usual effect of low temperature in lactic acid fermentation.

Due to the above factors, low temperatures are rarely of value for actual disinfection; in agricultural lactic acid fermentation, the effect of low temperature is usually limited to the decreased velocity and comparative inhibition of the lactic processes. The decrease in numbers of lactics during cold storage of many lactic acid fermentation media is not due to specific action of low temperature itself. In many cases, at least when the medium is not actually frozen, low temperature has a protective influence which is exhibited by a decreased death rate

coincident with the retardation of life activity. Moreover, under low temperature conditions, the specific action of most deleterious agents would be retarded and proceed at a slower rate than at usual temperatures.\*

It should be emphasized that the rôle of low temperature is that of a condition, not that of an active agent; it is a retarding influence which inhibits the action of harmful as well as beneficial reactions of the environment upon micro-organisms.

d. Indifference of lactic acid bacteria to sudden cooling.

This point is introduced merely to dispose of an empirical statement which has been made by a number of investigators. A few men have claimed that a sudden fall in temperature was accompanied by some deleterious effect upon bacteria. By them it was assumed that suddenly cooling material which had been held at a high temperature (as in processing of food or in thermal death experiments) caused the death of a certain number of the bacteria present. As early as 1897, Marshall showed that immediate cooling of laboratory pasteurized milk did not have any appreciable effect upon the micro-organisms present. This

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\* See End Part of "Lactic Acid Fermentation".

was confirmed by Ayers and Johnson in 1910 and since then, by a number of observers.

Jensen (1919), however, claims that in some cases even slight falls in temperature have a harmful effect upon some lactic acid bacteria. "We have also seen cases (e.g., with *Bacillus bulgaricus*) where a sudden fall in temperature of only 5° occasioned a serious weakening." This is the more surprising as it occurred with stock cultures kept at approximately 18°, in which case it would seem that a fall of 5° would tend to increase the keeping quality of the culture.

## II. Relation of Lactic Acid Bacteria to Desiccation.

### 1. Factors influencing resistance of lactic acid bacteria to desiccation.

Lactic acid bacteria are quite resistant to desiccation but their relative resistance is dependent upon many factors, which are discussed as follows, by Rogers (1914).

Among the conditions tending to destroy life during the desiccation process is the increase in ( $H^+$ ) resulting from the decrease in volume of the medium. Moreover, the increase in concentration of the solids in the water surrounding the cells may reach a point where plasmolysis of the cells results by withdrawal of their water content by osmotic pressure.

After the culture has been dried, or is in a state of desiccation, the following factors will largely determine how long it will retain its vitality: degree of dryness (or amount of moisture), temperature at which it is held, and possibly also the nature of the gas by which it is surrounded. (See "Oxygen".) The resistance to desiccation depends upon the ability of the cells to enter into a dormant state and still be able to regain activity when they are again introduced into a favorable milieu.

Thus, it may be stated that to give the organism longest resistance to desiccation it must be put under conditions in which its life processes are prohibited. When the moisture, heat, oxygen and food

(food prohibited by lack of water) conditions are least desirable for active growth, the organism will approach more nearly an absolute dormant condition. It is then that its actual death will be postponed the longest.

These factors are very important in the preparation of lactic cultures. They must also be kept in mind in the interpretation of the following investigations. In these experiments, the material on which the lactic acid bacteria were dried, as well as other of the above factors, will exert an influence upon results.

## 2. Examples of resistance of lactic acid bacteria to desiccation.

Kayser (1894) has exposed lactic acid bacteria dried upon strips of filter paper to desiccation for three months at 25° and lower temperatures, without observing loss in vitality. Troili and Peterson obtained similar results with lactic acid bacteria dried on silk threads for three and one half months. H. Weigmann (1898) has found living individuals in commercial cultures several years old. Von Freudenreich and Thöni claim that *B. lactic acid* resisted drying in a vacuum at room temperature for 312 days, which was the time of the last test. The same investigators found that at 37° the desiccated cells retained life for much shorter time,-- in the case of the above species, only 45-50 days. Other lactic acid bacteria were similarly affected by the rise in temperature --- strains of *B. casei* that resisted desiccation at room temperature for 67 and 135 days succumbed to exposure at 37° in 2 and 8 days. White and Avery found that their *Lactobacilli*, although not long lived in liquid media, retained vitality for four months when dried in a desiccator over sulphuric acid. Wehmer subjected *Lactobacilli* to desiccation under the following conditions: The organisms were grown in a flask in the presence of calcium carbonate. The cotton plugs were then carefully wrapped with paper and the lactic acid bacteria dried upon the precipitated calcium lactate. These organisms survived for surprising lengths of time; tests up to six years yielded actively fermenting lactic acid bacteria (a test made after ten years was negative).



From the above, it is evident that lactic acid bacteria are very resistant to desiccation under favorable conditions. This important property is of great advantage in the use of commercial starters for dairy products and for therapeutic purposes. It must be remembered, however, that a culture exposed to desiccation for a long period of time cannot be expected to set up as vigorous a fermentation as would a young actively growing culture. Besides this loss of activity, there occurs a gradual decrease in the number of living cells and the positive test obtained after desiccation for long periods are probably due to the survival of but a few cells, which may possess certain resistant characteristics.

### 3. Practical significance.

The relation of lactic acid bacteria to moisture (and factors as osmotic pressure,  $(H^+)$ , etc., included in conditions in concentrated media) is important also in the storage of butter, in the preparation of condensed milk and sauer kraut, as well as in the preparation of commercial lactic cultures.

### III. Osmotic Pressure and Concentrated Solutions.

Lactic acid bacteria are, of course, subject to the influence of this physical factor. However, in its study, it is extremely difficult to eliminate all other factors in order to determine the specific effect of osmotic pressure. The chemical properties



of the solute play an important rôle in determining the effect of concentrated solutions as well as does the osmotic pressure itself. Other factors also enter to complicate the process by which concentrated salt solutions exert their influence upon bacteria. For this reason, the rôle of osmotic pressure in the collective environmental effect upon lactic acid bacteria will be considered in greater detail later, in our discussion of the effect of different concentrations of salt.

Similar physical influences would be brought into play by equivalent concentrations of other electrolytes, but the presence of most ions would introduce other factors to a greater extent than do those of sodium and chlorine. Non electrolytes, such as sucrose, are used in many cases to obtain the advantages of a concentrated medium in food preservations. Naturally, higher concentration of non electrolytes are required to secure inhibitory osmotic pressure conditions.

As the pneumococcus is more or less closely related to at least some strains of lactic streptococci, the following investigation of Demby and Avery is of interest here. They investigated the influence of different molar concentrations of potassium chloride upon the growth of this organism in broth. They found that concentrations up to 0.1 were without effect upon the pneumococcus; that concentrations of 0.2 retarded growth; and that concentrations of 0.4 and higher prohibited growth. They concluded that the addition of salts to media for growth of the pneumococcus, (such as phosphates for buffer effect), should be limited to concentrations not over 0.1 m.

A comparison of the salt effect on the resistance of pneumococcus with that of common lactic acid bacteria is afforded by referring to the discussion of the influence of high concentrations of salt.

#### IV. Light and Other Rays.

##### 1. Probable insignificance of this influence in lactic acid fermentation.

The influence of light rays upon the microbial agents of agricultural lactic acid fermentation is not very important outside of its purely academic interest. In their natural media, the lactic acid bacteria are not exposed to high intensity of light for a long enough period for this physical factor to be of moment. The significance of the action of light rays in the agricultural rôle of lactic acid bacteria is probably limited to such partial sterilization of empty milk cans and the containers of other lactic acid fermentation media as might possibly occur when these are placed in direct sunlight for several hours. Due to its incomplete effect and the efficiency of other methods, this incident is of little importance.

*B. coli* has been the subject of investigations on the effect of light upon micro-organisms merely because it is a common, well known species, and not because of any reference to lactic acid fermentation. It has been found to be not particularly susceptible to the action of sunlight. (Rahn, 1917)

## 2. Experiments of Richet with different rays.

Richet has used lactic acid bacteria in interesting experiments in the investigation of the relation of certain light rays to micro-organisms.

He placed glass ampules containing the phosphorescent sulphide of calcium into milk cultures of lactic acid bacteria. He found that there was produced an initial stimulation, as indicated by increased acid production over the controls. This was followed by a retardation of activity.

He did not believe this influence could be due to the faint luminosity afforded by the presence of the phosphorescent sulphide and, while reaching no definite conclusion, he thought it possible that  $\gamma$  rays were involved.

In later investigations, Richet (1906) again used lactics as a convenient index of the action of radium rays upon micro-organisms. He found that they exerted a certain influence upon these lactic acid bacteria.

## 3. Action of ultra violet rays.

In their investigation of the use of ultra violet rays in milk sterilization, Ayers and Johnson (1914) found that the lactic acid bacteria in milk were greatly reduced in numbers by the action of these rays. Their action was not a specific bac-

tericidal effect upon lactics; spores in milk were more resistant and the authors believe action upon lactics was largely due to the greater susceptibility of vegetative cells. Houghton and Davis have also investigated germicidal action of ultra violet rays as a means of sterilizing or pasteurizing milk. They claim that their results indicate "that exposure of milk to ultraviolet rays has a tendency to kill off undesirable organisms present, leaving more desirable bacteria of *Bact. lactis acidi* group in the majority." They found mold spores were hardly affected by this treatment. Although they found that this group of lactics predominated after exposure of the milk to the rays, their conclusion intimating greater resistance of these organisms is hardly warranted.

## V. Mechanical Effects.

### 1. Agitation.

Gentle agitation is said to have a stimulating effect upon micro-organisms (Rahn, 1917). Gutzeit, (1911), has reviewed the influence of this factor upon lactic acid bacteria.

In lactic acid fermentation, the importance of this factor is probably limited to the breaking apart of clumps and colonies, with the possible sequence of stimulated growth activated and fermentation due to the removal of the micro-organisms from a

sphere of relatively higher local concentration of metabolic products. This may be of some significance in certain processes of handling milk. (Marshall and Hood, 1918)

## 2. Gravity and centrifugal force.

Centrifugal force may at times exert much the same influence upon lactic micro-organisms as that suggested for simple agitation.

More often, however, in agricultural lactic acid fermentation a larger part of the moment of this physical factor may be attributed to a disturbance in the flora equilibrium and consequent changes in microbial associations. (Marshall and Hood, 1918).

## 3. Mechanical pressure.

Although the influence of this factor has few, if any, applications, in the usual lactic acid fermentation, pressure has been shown to have a certain effect upon lactic acid bacteria.

Hite, Giddins and Weakley (1914) investigated the ability of several micro-organisms to withstand high pressures. Although their results are rather variable, the following data show that bacteria are very resistant to pressure under the conditions of their experiments. *Lact. lactic aerogenes*, suspended in distilled water: killed by momentary exposure to 90,000 pounds, or by ten minutes' exposure to 65,000 to 80,000 pounds; a few cells survived throughout tests for 110-130 minutes at 30,000 pounds; all cells were killed in 150 minutes at 30,000 pounds.

*Strep. lacticus*, suspended in 3% lactose broth: slight to good growth after momentary exposure to 100,000 pounds; sometimes all cells killed by 5



minutes' exposure to 90,000 pounds; killed by 10 minutes, at 60,000 pounds; survived 150 minutes, at 40,000 pounds.

Although very high pressures are required for a lethal effect, lower pressures will inhibit growth. Lactic acid fermentation has been shown to be delayed in milk kept under high pressure.

Rahn (1917) explains the influence of pressure upon growth of lactic acid bacteria largely upon the factor of oxygen concentration relations following from Henry's law. It is just as probable, however, that the influence of pressure is a cumulative effect and that micro-organisms possess cardinal points in their pressure as in their temperature relations --- the point of inhibition of multiplication would naturally be lower than that of germicidal effect.

In a study of its effect upon micro-organisms, many factors must be considered. Among these are the medium upon, or in which the bacteria are subjected to the pressure; the solubility of gases present in the system; and other factors, such as arise from the principles of Dalton's law of partial pressures and of Henry's law.

Chapin and Tamman (1903) furnish a historical survey and a discussion of the influence of pressure upon micro-organisms.

## B. BIOCHEMICAL INFLUENCES

### I. Influence of Carbohydrates.

1. Rôle of carbohydrates in the metabolism of lactic acid bacteria.
  - a. Source of energy.
  - b. Source of carbon.
2. Influences exerted upon lactic acid bacteria by different carbohydrates.
  - a. Differences in availability.
  - b. Differences in products yielded.

### II. Influence of Nitrogenous Materials.

1. Sources of nitrogen.
2. Favorable influence of "peptone" upon the medium.
  - a. Reports upon concentration of "peptone".
  - b. Reports of preference for kind of peptone.
  - c. Reports of "acclimatization" of lactic acid bacteria in regard to the source of "peptone".

### III: Influence of Oxygen Concentration.

1. Limitation of discussion.
2. Earlier reports of the relation of oxygen to the life of lactic acid bacteria.
3. Variation among different lactic acid bacteria.
4. Oxygen concentration relations of different groups of lactic acid bacteria.
5. Influence of oxygen concentration upon products of growth.
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#### IV. Influence of Hydrogen Ion Concentration upon Lactic Acid Bacteria.

1. Early reports of influence of acids.
2. Michaelis and Marcora's interpretation upon basis of effect of hydrogen ion concentration.
3. Hydrogen ion concentration zones as limits of growth for lactic acid bacteria.
  - a. Comparison of the two standpoints from which  $pH$  toleration of bacteria is considered.
  - b. Reports on  $pH$  toleration of different lactic acid bacteria.
    - (1) Acid gas group.
    - (2) Strep. lacticus group.
    - (3) Lactobacilli.
    - (4) Fourth group.
  - c. Importance of  $pH$  toleration by lactic acid bacteria.
4. Adjustment of hydrogen ion concentration to zones favorable to lactic acid bacteria.
  - a. Effect of adjustment.
  - b. Means of adjustment.
    - (1) Chemical.
    - (2) Biological.
5. Factors conditioning the moment of the hydrogen ion concentration factor.
  - a. Different systems.
  - b. Different influences upon different cell functions.
6. Optimum hydrogen ion concentration for lactic acid bacteria.

## V. Influence of Other Chemical Substances.

1. Salts of the metals or metallic ions --- general agreement with the M. M. F. series.
2. Ions exerting a selective action on lactics.
  - a. Zinc ion.
  - b. Fluoride ion.
  - c. Phosphates.
3. Common disinfectants.
4. Lecithin.
5. Carbon dioxide and other gases evolved during putrefaction.

## VI. Influence of Different Concentrations of Salt.

1. Influences operating.
2. Effect upon other conditions in the system.
3. Effect upon microbial balance.
4. Relation of different lactic acid bacteria to various concentrations of salt.
5. Significance in agricultural lactic acid fermentations.

## INFLUENCE OF THE ENVIRONMENT UPON LACTIC ACID BACTERIA

### B. BIOCHEMICAL INFLUENCES

#### I. Carbohydrates.

##### 1. Rôle of carbohydrates in the metabolism of lactic acid bacteria.

The carbohydrate presented by the medium to lactic acid bacteria serves two principal purposes; that of a source of carbon and of a source of energy. The relative importance of these to the lactic has been discussed before. There it was shown that a small part of the carbohydrate may be diverted to uses of the cell. Jensen (1919) claims that this part of their food is used particularly in the building of cell walls. The portion of the total carbohydrate consumed, which is diverted from the main reaction for this purpose is very small.

A large amount of energy can be derived from a fermentable carbohydrate compared with other constituents of a medium. They are also usually more immediately available to the lactic micro-organism. For these reasons, the principal rôle of a carbohydrate in lactic metabolism is as a source of energy. The much more vigorous growth of lactic acid bacteria in media offering a fermentable sugar is evidence of the preference exhibited by lactic acid bacteria for these substances as their source of energy.



2. Influences exerted upon lactic acid bacteria by different carbohydrates.

a. Differences in availability.

The carbohydrates available have been given under a discussion of substrates of the enzymes of lactic acid bacteria. Even among carbohydrates ultimately available to their enzymes, differences are observed in the readiness with which they are attacked by the lactics. Some of the carbohydrates which may be fermented by a lactic growing under the best conditions may not be attacked at all in systems less favorable. (In case of polysaccharides, this may involve the elaboration of hydrolytic enzymes.) Again, differences are frequently observed in the rate of acid formation from different sugars, or in the velocities of the action of the enzymes of lactic acid bacteria upon different carbohydrate substrates.

It is generally assumed that a hexose offers the most available source of energy to lactic bacteria. As a rule, the six-carbon sugars are more easily fermented than are the disaccharides and more complex carbohydrates. However, this need not always be the case.

Jensen (1919) points out that there is "nothing to prevent the (fermentable) disaccharides from being better nutriment than the monosaccharides of which they are composed." He shows that certain of his "betacocci" seem to prefer sucrose to either glucose or fructose.

A streptococcus from sauerkraut, which seems to resemble Jensen's "betacoccus" group, prefers sucrose to glucose, as evidenced by its more rapid acid formation from that substrate (see Part II).

The hexoses differ among themselves in their availability.

Jensen (1919) reports that galactose, as a rule, is the hexose most difficultly fermented by lactic acid bacteria. He observed that some strains preferred levulose to dextrose. Fred and associates (1920) believe that fructose is more rapidly attacked by their pentose fermenting bacteria than are the aldoses.

#### b. Differences in products yielded.

The fermentation of different carbohydrates by lactic acid bacteria may yield different products. These differences may be not only quantitative but also qualitative.

Reports of production of different amounts of acid by the fermentation of different sugars by the same lactic are frequent in the literature. It is interesting to note that quite often the largest yield of acid is obtained from the fermentation of carbohydrates less readily attacked by the fermenting agent. The introduction of ( $H^+$ ) measurements at the end point of fermentation shows that differences also exist in the final pH value reached in the lactic acid fermentation of different carbohydrates.

(These points will be referred to in the discussion of the "Theoretical Progress of Lactic Acid Fermentation.")

The influence of the carbohydrate sometimes decides the direction as well as the extent of a fermentation. This is clearly shown in the discussion of the influence of the substrate upon "Other Products of Lactic Acid Fermentation".

In addition to the examples given under that heading, the following show that the fermentation of different carbohydrates may yield wholly different products.

Jensen (1919) reports that his "betacocci" produce slime from sucrose, but not from glucose or fructose. Fred and associates (1920) found that the aldoses yielded different products from those yielded by fructose, when fermented by the pentose fermenting lactobacilli. This later observation has frequently been made in the case of acid fermenting bacteria. It is evident in the work of Smit given below.

Among others, Smit (1915) has reported examples of the influence of the substrate upon the products of fermentation. The following table represents the relative amount of the various products yielded in the fermentation of glucose, fructose, and sucrose by *Lactobacillus fermentum*.

	Dextrose	Levulose	Sucrose
Carbon dioxide	14.1%	7.0%	17.4%
Alcohol	16.9	---	16.8
Lactic acid	47.1	12.3	33.7
Acetic acid	3.7	12.9	6.1
Formic acid	0.1	0.2	0.1
Succinic acid	1.2	1.4	0.9
Mannitol	---	60.1	23.8
Glycerol	6.3	---	---

The integral value of such a table is however, severely conditioned by the possibility of secondary reactions upon the initial products of the substrate. In many such cases, the relative amount of the various products present in the fermentation system is constantly changing, at least during the earlier periods of the fermentation. Some of the initial products of the original substrate will be attacked by other more or less simultaneous reactions, whose velocities will also be constantly changing. (Compare "Alkaline fermentation of Acid Salts" in "Theoretical Progress lactic acid fermentation".) In addition to these simultaneous reactions, the first formed products are often attacked by delayed (probably purely enzymatic) reactions, which are not apparent until long after cessation of active growth of the fermenting agent.

These suggestions may apply to Smit's determinations, particularly in the case of carbon dioxide, formic acid, and mannitol. (See "Other Products".)

## II. Influence of Nitrogenous Materials.

### 1. Sources of Nitrogen.

The ability of these substances to serve as sources of nitrogen will depend to no small extent upon the other food constituents and environment in which they are presented. They are usually presented in media containing a readily fermented sugar, for reasons given in the discussion of carbohydrates. The fact that these substances cannot serve as a source of nitrogen in media containing no other nitrogenous material need not preclude the possibility of their utilization in other media as a partial source of nitrogen. These facts should be kept in mind in the interpretation of all of the following reports.



a. Ammonium salts, acid amides and amino acids.

Fränkel obtained growth of *Bac. acidilactici* (*B. aerogenes*) and several other organisms in media in which asparagine was the source of nitrogen. Hueppe found the same organism using ammonium tartrate. Kozai found some lactic acid bacteria grew in asparagine media, but was unable to obtain growth of a lactic probably identical with *Strep. lacticus*.

The more recent investigations of Clark and Lubs (1917, a) and of Ayers and Rupp (1918) show that the acid gas group are able to grow in media containing nitrogen in the form of sodium ammonium phosphate.

The latter (1918, b) have suggested a synthetic agar for "direct count" of the colon-aerogenes group, in which the above salt is the source of nitrogen. The selective action of this medium is based on the ability of the acid gas group to obtain nitrogen from the ammonium salt and the failure of other lactics to utilize this salt.

Jensen (1919) claims that amino acids alone cannot serve as a source of nitrogen for "true" lactic acid bacteria and limits the formation of ammonia by these organisms to that split off during the hydrolysis of protein molecules.

b. Peptides and other constituents of "peptone".

Certain of these substances seem to offer the most suitable source of nitrogen to lactic acid bacteria. Probably, the simpler of the "peptone" constituents are preferred. Much of the effect of

increasing the "peptone" content of media is probably due largely to increases in concentration of certain preferred nitrogenous foods. This question will be discussed again in the reports of investigations on the influence of "peptone" upon lactic acid bacteria.

These substances are also able to serve as sources of energy to lactic acid bacteria, as evidenced by the growth of lactics in "peptone" media, in the absence of fermentable sugar.

### c. Proteins.

It is doubtful whether proteins ever serve as sources of nitrogen for many types of lactic acid bacteria, although this may possibly occur with certain lactobacilli and members of the fourth group. At least in the case of the more common lactics, the attack upon casein may be considered as a purely enzymatic action, as a significant proteolysis of casein is not observed until a period in which its endoenzymes are liberated by autolysis. (See also "Enzymes".)

## 2. Favorable influence of "peptone" upon the medium.

### a. Reports upon the concentration of "peptone".

Many reports of the favorable influence of increased "peptone" concentration upon lactic acid bacteria are reported in the literature. Most of

these experiments have consisted in the addition of simpler non-protein substances to media initially poor in non-protein nitrogen. This influence is readily understood by considering the relation of lactic acid bacteria to these substances, as reported above and under our discussion of "Enzymes".

Kayser (1915), Jensen (1898, 1915), Hastings, Evans and Hart, Koestler (1907), and many others have demonstrated increased activity of lactic acid bacteria upon increasing the peptone content of the media. Using peptonized milk as a medium for lactic acid bacteria, Jensen (1898) obtained increased acid production over that obtained in milk in which the casein was not hydrolyzed. (Von Dam has criticized Jensen's conclusions, pointing out that, at least a certain part of the favorable influence of peptone may be due to its buffer action. There is much truth in his statement, "The question of direct influence of peptone is not to be determined without a consideration of the buffer action of this substance.")

Because of the difference in titratable acidity resulting from presence of larger concentrations of peptone, the influence of this factor should perhaps be judged upon the basis of its influence upon multiplication of cells.

Upon this basis, Rahn (1911) has considered the results of many experiments of his own and of Marshall and associates. He found (as had Jensen, Kayser, and Koestler, upon the basis of acid produced), that different lactics are influenced differently by the peptone concentration of the medium. While some do not respond, other strains show a decided increase in multiplication. He found that, in case of many strains, growth of lactics in milk is apparently conditioned by the concentration of simpler nitrogenous substances of that medium. He ascribed the checking of growth in usual milk cultures of lactics to the diminished concentration or removal of the utilizable nitrogenous food. (See "End Point of Lactic Acid Fermentation.")

The favorable influence of Marshall's associate peptonizers, possibly also Northrup's associate red yeasts; the enrichment media so advantageous in the cultivation and isolation of lactic acid bacteria, such as Jensen's and Evans' peptonized milk, Rubnisky's yeast extract whey, Löhnis' peptone-fortified whey and milk --- all give further evidence of the favorable influence of a peptide-like source of nitrogen upon the life processes of lactic acid bacteria.

b. Reports of preference for kind of peptone.

The above discussion has shown that lactic acid bacteria are influenced by concentration of "peptone" and that their requirements are best met by a medium of high peptone content. Various observers have reported that lactic acid bacteria prefer certain kinds of "peptone". This also should be considered in the light of above statements upon the variety of protein-hydrolytic products included under that term. Moreover, it must be remembered that many lactic acid bacteria prefer certain of these constituents to other probably more complex combinations.

Jensen (1913) claimed that casein peptone is a more favorable source of nitrogen for the "true" lactic acid bacteria than is fibrin peptone. This may be due to presence of the different actual constituents in concentrations varying with the source and degree of hydrolysis of the so-called "peptones". In his latest report (1919) he emphasizes the differences in value to lactic acid bacteria, exhibited by different "peptones". Casein peptone he found more



favorable than Witte's peptone in all cases. The value of yeast extract varied with the species, being particularly favorable to the lactobacilli. He also emphasizes the different buffer action of different sources of nitrogen. He reports that the buffer value of Witte's peptone, casein peptone and yeast extract increased in the order given. He found that yeast extract broth (as made by him), containing 0.5 % nitrogen, had about the same buffer action as Witte peptone broth containing 1.35 % nitrogen (i.e., 10 % Witte peptone). However, he does not believe that all of the differences in the influences of different peptones can be ascribed to their buffer action.

Much of the difference in the relation of lactic bacteria to different peptones is due to the degree to which the product has been hydrolyzed. Avery and Cullen (1920) have shown that the enzymes of pneumococcus attack the simpler peptides of Fairchild's peptone with greater avidity than Witte's more complex product.

c. Reports of "acclimatization" of lactic acid bacteria in regard to source of "peptone".

Hennenberg has found that lactic acid bacteria from milk and milk products do not grow well in beer, while lactic acid bacteria of the brewery and distillery grow well in beer but slightly or not at all in milk. Jensen (1919) reports some types of lactic acid bacteria grow poorly in milk, but well in yeast extract. A sauerkraut streptococcus studied in this laboratory grows better in lactose than in milk.

Such examples of apparent acclimatization of certain lactic acid bacteria to different nitrogenous food should be so interpreted only after careful study. These may be nothing more than manifestations of definite differences in the availability of the different constituents of these media.



### III. Influence of Oxygen Concentration

#### 1. Limitation of discussion.

The relation of oxygen to the chemical reactions of lactic acid fermentation has been discussed before, under "Chemical Changes". This discussion is concerned with the relation of oxygen to the growth and functioning of lactic acid bacteria. Mayer's "cardinal points" apply to the oxygen concentration requirements of all micro-organisms. The following discussion is concerned with the optimum oxygen concentration for lactic acid bacteria.

#### 2. Earlier reports of the relation of oxygen to the life of lactic acid bacteria.

The question of the relation of lactic acid bacteria to oxygen, (or their oxygen concentration requirements), has been much disputed among the earlier investigators.

Hueppe and Richet (1892) claimed that an abundance of oxygen exerted a favorable influence. Others, as MacDonnell and Troili and Peterson claimed that oxygen was without influence. Others, as Epstein and Mayer later asserted that it was under an anaerobic condition that the lactic acid bacteria were most active.

#### 3. Variation among different lactic acid bacteria.

The experiments of Kayser (1894) and later, those of Koestler explain these contradictory claims. The results of Kayser's experiments led him to conclude that different lactic acid bacteria have different oxygen requirements; some grow better under aerobic conditions, others under anaerobic conditions,

while still others are apparently indifferent to the presence of oxygen.

4. Oxygen concentration relations of different groups of lactic acid bacteria.

The four groups of lactic acid bacteria seem to require different amounts of oxygen for optimum growth. The acid gas group probably demand higher oxygen concentration for their maximum activity than do the second and third groups, but even these organisms are able to grow in its absence, if a fermentable sugar is present.

The *Strep. lacticus* group exhibit a lower optimum oxygen concentration and seem to function at least as well in its absence as in its presence.

(The above reports of MacDonnell and of Troili-Peterson were concerned particularly with this group). In the investigation to be reported in Part II of this paper, a lactic of this type showed greater proteolytic activity in the absence of oxygen. Heinemann, on the other hand, reports greater activity of *Strep. lacticus* in the presence of free oxygen. Not only was more acid formed under aerobic conditions, but a larger number of carbohydrates were fermented than was the case under anaerobic conditions. The usual variation among strains probably extends to their oxygen relations, but it seems that most members of the *Strep. lacticus* group grow better under low oxygen concentration conditions.

In the group of lactobacilli are found lactic acid bacteria functioning best in very low oxygen concentrations. The experiments of Koestler were concerned principally with this group. He found that there were not only differences between strains in their oxygen relations, but that the ef-

fect of different oxygen concentrations upon the same organism varied with other conditions, such as temperature and peptone content of the medium.

The fourth group is such a heterogeneous collection that no definite statement can be made concerning their oxygen relations; it may safely be stated, however, that most of these organisms have higher optimum concentrations than members of the second and third groups.

Those of Jensen's (1919) lactic acid bacteria, which correspond most closely to this group, were found to have higher oxygen concentration requirements than did most of his types.

#### 5. Influence of oxygen concentration upon products of growth.

The results of many investigations indicate that in presence of oxygen a greater proportion of volatile acids is formed than in its absence (see "Other Products"). In mixed cultures this could be explained as the result of the acid gas group, (which, it will be remembered, form large amounts of volatile acids and which probably are also favorably influenced by the presence of oxygen), gaining ascendancy in the medium. In other cases it may be due to a combustion of the lactic acid itself, which reaction would probably proceed to best advantage under aerobic conditions.

#### 6. Other effects of high oxygen concentration upon lactic acid bacteria.

The above discussion of the oxygen relations of lactic acid bacteria has been limited to oxygen concentrations within the range of growth of microorganisms. This, however, is a very limited range, as is evident from a consideration of the solubility of this gas and the fact that only dissolved substances enter microbial cells. No evidence is at hand concerning the effect of higher oxygen concentrations upon lactic acid bacteria, other than the well known poisonous effects of slightly higher oxygen concentrations than those included in their range of growth. Much of the apparent effect of pressure upon microbial life may be assigned to changes in the oxygen concentration of the medium (Rahn). The mode of the disinfection effect of desiccation is also ascribed to action of oxygen. Death by drying is said to be an oxidation process by which fact may be explained the increased rate of death when desiccated cells are held under high oxygen concentration conditions.

#### IV. Influence of Hydrogen Ion Concentration upon Lactic Acid Bacteria.

##### 1. Early reports of influence of acids.

The effect of acids upon lactic acid bacteria was observed by the early workers on lactic acid fermentation. In 1841 Fremy and Boutron Chalarid ascribed the cessation of action of their "ferment lac-

tique" to the harmful influence of the lactic acid present in the medium. Others of the early investigators made similar observations which were in accord with the general understanding that an accumulation of the products of a fermentation soon inhibited the activity of the ferment. However, it was soon seen that the influence of acids upon lactic acid bacteria was not limited to those produced during the fermentation. It was found that mineral acids exerted a still more harmful influence than did lactic acid. Weigmann (1910) reports early investigations showing inhibition of lactic acid bacteria by 0.07% to 0.08% hydrochloric acid and 0.04% sulphuric acid.

The relative influence exerted by different acids and the harmful influence of all acids were explained more fully by the introduction of physical chemical methods into the investigations of the biology of lactic acid bacteria.

2. Michaelis and Marcora's interpretation,  
upon the basis of the effect of  
Hydrogen ion concentration.

In 1912 Michaelis and Marcora explained this question in a convincing and illuminating manner.

They grew the lactic acid bacterium, *E. coli*, in lactose broth, to which varying amounts of sodium hydroxide had been added. Notwithstanding the difference in initial alkalinity, the concentration of the hydrogen ion at which the life processes of the lactic acid bacterium were inhibited was always practically the same. Naturally, different amounts of acid would have to be introduced in order to bring these broths of different initial alkalinity up to this ( $H^+$ ).



Therefore, they concluded that the harmful effect of the acid was due merely to the dissociation of its cation, as lactates in moderate concentration were apparently harmless.

This work explained the harmful influence of acids, not upon the amount of acid introduced, but upon the hydrogen ion concentration, which is the basis upon which this environmental factor will be discussed in the following paragraphs.

### 3. ( $H^+$ ) zones as limits of growth for lactic acid bacteria.

From their results, Michaelis and Marcora concluded that *B. coli* was inhibited in hydrogen ion concentration above pH 5. The work of these men has been extended by other investigators to so great a number of species and strains of bacteria, (many of them lactic acid bacteria), that it may be regarded as established that all bacteria are limited to fairly definite zones of hydrogen ion concentration toleration and that, under like conditions, these zones of hydrogen ion concentration toleration are practically a physiological constant for many of the different species and strains of lactic acid bacteria.

#### a. Comparison of the two standpoints from which pH toleration of bacteria is considered.

The ( $H^+$ ) tolerance of bacteria has been developed from two aspects. Working with proteolytic organisms, Itano determined the pH range of actual growth by inoculating vigorous cells into media of a

progressive series of  $pH$  values. Clark and associates have been concerned with organisms of distinctly acid forming proclivities and have developed their method of determining the ( $H^+$ ) relations of these organisms upon basis of final  $pH$  produced in sugar broths.

These interpretations are slightly different: the fermentation limit of Clark is not necessarily the same as the upper limit of Itano's  $pH$  range of growth.

This is evident from the intensive study of Avery and Cullen (1919) upon the ( $H^+$ ) relations of pneumococcus. They found that it is impossible to initiate growth of this organism in media having a more acid reaction than  $pH$  6.8, although the pneumococcus will exhibit a fermentation limit of  $pH$  5. "For instance, if the organisms are alive and growing at  $pH$  5.5, and a seeding is removed at this point and implanted in a medium with a reaction of  $pH$  6.5, no growth occurs." Avery and Cullen would term the  $pH$  zone at which the micro-organism is able to initiate growth as the "limiting initial ( $H^+$ ).". The same phenomenon is exhibited by at least four types of streptococcus, although with these organisms, the "limiting initial ( $H^+$ )" agrees more closely with their final "fermentation limit" than in the case of pneumococci. (See Part II of this paper.)

The lactic acid bacteria have been investigated chiefly from the standpoint of the fermentation limit, --- the ( $H^+$ ) of the medium at the end point of the fermentation. As a measure of acid production of these and other organisms, the determination of this point has now supplanted the former titrimetric determinations. The "end point of lactic acid fermentation" (see "Theoretical Progress of Lactic Acid Fermentation") is concerned largely with the "fermentation limit"  $pH$

zones of lactic acid bacteria.

b. Reports on pH toleration of different lactic acid bacteria.

(1) Acid gas group.

Clark and Lubs (1916) later found about the same limiting ( $H^+$ ) zone for a large number of strains of *B. coli* as that found by Michaelis and Marcora. Winslow and associates report a similar fermentation limit for the acid gas groups studied by them.

The fermentation limit of the aerogenes group must not be confused with the final ( $H^+$ ) reached under the conditions of the methyl red test, as with these organisms the final pH value is very dependent upon other reactions. Recent work by Cohen and Clark indicates that, under certain conditions, these organisms can reach as high ( $H^+$ )'s as the *B. coli* or "methyl red +" group.

(2) *Strep. lacticus* group.

Itano (1916) found the ( $H^+$ ) range of growth of *Strep. lacticus* in peptone sugar free broth to be between pH 4.7 and pH 9.5. Evans has shown that the final ( $H^+$ ) reached in different sugar broths by different strains of *Strep. lacticus* varied from pH 4 to pH 5. Van Slyke and Baker found the fermentation limit in milk for other strains of this lactic to be between pH 4.17 and pH 4.56.

(3) *Lactobacilli*.

As *lactobacilli* are known to grow in media of higher ( $H^+$ ) than do most members of the other groups, it is safe to assume that they tolerate still higher ( $H^+$ )'s.

Clark found that one strain of *B. bulgaricus* had a fermentation limit of pH 3.9. Van Slyke and Baker found that one strain of *B. bulgaricus* reached pH 3.7 in milk incubated at 25° C.. Fred and associates report still higher fermentation limits in case of their pentose fermenters. These lactobacilli reach a pH of 3.0 in certain sugar media. They also exhibited a wide range of pH tolerance as they were able to grow in media varying from pH 3.0 to pH 8.6.

#### (4) Fourth group.

The fourth group probably exhibit a still greater variation in their final ( $H^+$ ). Certain cocci studied by R. C. Avery in this laboratory, (which probably belong to this group), show final end points approximating and often exceeding those of the lactic streptococci.

##### c. Importance of pH toleration by lactic acid bacteria.

The pH fermentation limit or the range of ( $H^+$ ) within which the life processes of the different lactic acid bacteria will proceed is of great importance. Many times this is the determining factor of the duration of lactic acid fermentation\* --- the medium may still contain plenty of available nitrogenous material and the fermentable carbohydrate may not be exhausted, but the lactic acid bacteria present are unable to function in the ( $H^+$ ) produced in the medium. (See "End Point of Lactic Acid Fermentation")

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\* See page "End Point", under "Progress of a Lactic Acid Fermentation".

#### 4. Adjustment of ( $H^+$ ) to zones favorable to lactic acid bacteria.

##### a. Effect of adjustment.

In such cases, if means are provided to lower the ( $H^+$ ) to a zone in which the lactic acid bacteria can carry on their life processes, growth and functioning of the lactic acid bacteria will continue, even though they have already produced an amount of acid which would prohibit their growth in unbuffered media. (This is, of course, limited to a certain extent by other factors to be discussed later under "End Point of Lactic Acid Fermentation").

##### b. Means of adjustment.

###### (1) Chemical.

In the early days, Fremy and Boutron Charlard observed the effect of adding neutralizing substances to the medium in which their "ferment lactique" was growing. They found that by this means the fermentation, which had been arrested by the acid present, proceeded up to the point of using up not only all the sugar present in the milk, but even added amounts. As early as 1893, Timyze ascribed the formation of larger amounts of acid in the lactic acid fermentation of milk than in that of sugar solutions to the more prolonged activity of the lactic acid bacteria because of the protection which was furnished by the acid combining power of the casein. It has long



been a common laboratory practice to add calcium carbonate or similar substances to cultures of lactic acid bacteria in order to permit their life processes to go on for a longer time.

For the same purpose, Henderson and Webster added phosphates to regulate the acidity and found that the life processes of the lactic acid bacteria (B. acidii) proceeded to better advantage in the buffered medium. Since then the work on the relation of ( $H^+$ ) to the biology of the lactic acid bacteria has explained this beneficial effect of neutralizing or buffer substances in the medium.

Von Dam goes so far as to attempt to explain Jensen's report of increased activity of lactic acid bacteria in peptonized milk as the result of the increased buffer effect of the medium. He gives tables and curves showing that the addition of the same amount of lactic acid to peptonized milk, peptone whey, and whey, produces the following ( $H^+$ )'s:  $1 \times 10^{-5}$ ;  $2 \times 10^{-5}$ ;  $6.6 \times 10^{-5}$ . As this buffer action would permit a larger amount of lactic acid to be formed before the limiting ( $H^+$ ) would be reached, it is not surprising to find that lactic acid bacteria produce more acid in a medium possessing such marked buffer action. Although the favorable influence of the simpler peptide nitrogen upon the lactic acid bacteria is definitely established, the examples cited show that "in a study of the biological characters of the lactic acid bacteria, the buffer action of the medium must be taken into consideration."

Substances used for neutralizing or regulating the ( $H^+$ ) must be limited to those not yielding ions antagonistic to lactic acid bacteria. (See following topic.)

Carbonates or phosphates of the alkalis or alkali earths are usually employed. Those of the heavy metals are undesirable. Since the work of Hen-

derson and Webster, polybasic phosphates have been used extensively as buffer substances in biochemical tests of acid production of the lactic acid bacteria, as in the methyl red test, etc.. Peptone, casein and other substances present in the media of many agricultural lactic acid fermentations also have the power of removing part of the acid produced from the action on the bacteria.

## (2) Biological adjustment.

Although a more extensive discussion of this question comes more appropriately later in this paper, it is interesting to note here that, (in addition to the buffer action of the above substances, which may be either present in the natural medium or added by design), some lactic acid bacteria seem to be able to regulate the ( $H^+$ ) of the medium by means of their own life processes.

Early investigators reported that the lactic acid bacteria seemed to consume part of the lactic acid produced, as evidenced by lower acidity of the medium. Later investigators would offer a different explanation of at least some of such cases. Clark and Lubs believe that many times this neutralizing action is more apparent than real, due to errors introduced by titration. The production of ammonia from result of action on peptones might in some cases account for the neutralization of a part of the acid produced (Berman and Rettger (1914), Levine (1916)). Ayers and Rupp show that, with at least some lactic acid bacteria, alkaline fermentation of salts of the organic acids produced is a potent factor in the neutralization of the acids and a consequent decrease in ( $H^+$ ). (See "Methyl Red Test").

The alkaline fermentation of organic salts and acids probably occurs in the media of many agricultural lactic acid fermentations. The consequent adjustment in many cases need not be brought about by the organism responsible for the

original production of the organic acids, but may depend upon the life processes of micro-organisms associated with them. (Compare "Associative Influences").

#### 5. Factors conditioning the moment of the $(H^+)$ factor.

The moment of the  $(H^+)$  factor in the environment of micro-organisms is emphasized, but its influence upon their various activities should be interpreted only with due consideration of the many other environmental conditions and agents.

The specific effect of the hydrogen ion is conditioned by many factors, such as the presence of buffer substances, temperature, food supply, and others. Its influence upon micro-organisms may often be altered by the presence of foreign ions, and in other cases, by the radical of the dissociated acid and even the undissociated acid. The influence of the foreign ions, acid radicals and undissociated acids may be due either to their specific action or to a synergic action upon the influence of the hydrogen ion itself.

Media of the same pH value, obtained by the addition of different acids, may have different influences upon certain micro-organisms.

Cohen and Clark have shown recently that *E. coli* tolerates higher hydrogen ion concentrations in media adjusted by hydrochloric acid than in those in which the pH value is due to the presence of acetic acid.

Recently it has been shown that differences may exist in the specific effect of the same hydrogen ion concentration upon the different activities of bacteria during their life history. (Cohen and Clark, Itano and Neill). In the case of lactic acid bacteria, the hydrogen ion may exert a distinctly different influence upon multiplication, upon the fermentation process itself and upon the end point of the fermentation (Cohen and Clark).

Further data concerning the significance of ( $H^+$ ) in the determination of the end point of lactic acid fermentation will be given in the discussion of that question.

#### 6. Optimum ( $H^+$ ) for lactic acid bacteria.

Determinations of the optimum ( $H^+$ ) for micro-organisms is more difficult. Probably different functions of the cell vary in their optimum pH requirements. Again, different enzymes of the cell require different ( $H^+$ )'s for their optimum activity. From these considerations (and there are still others) it is seen that determinations of optimum pH for an organism will vary with the index chosen for its measurement.

Several observers have attempted to measure the optimum pH for growth of certain lactic acid bacteria. Here the optimum pH zone may be considered as the optimum ( $H^+$ ) for those processes of the cell which are involved in multiplication.



In the case of bacteria more sensitive to ( $H^+$ ), investigations can be made on the effect of small increments of  $pH$  (Demby and Avery, on the pneumococcus). With the more common lactic acid bacteria, such determinations can usually be made only for comparatively wide zones.

In the case of the lactic streptococcus, Itano (by turbidity) and Svanberg (1919) found optimum growth to occur in media of about  $pH$  6.0.

This question is investigated in Part II of this paper, in the case of different types of streptococci.

Studies of the optimum  $pH$  for certain functions of lactic acid bacteria are usually determined by measurements of their products. Here, one is concerned principally with the effect on the enzymes involved.

Itano has investigated the optimum  $pH$  for proteolysis of *Strep. lacticus*. He found that the greatest accumulation of amino acids occurred in broth having an initial ( $H^+$ ) of between  $pH$  6.0 and  $pH$  7.0. This point is again investigated in the case of different types of streptococci in Part II of this paper, where a further discussion will be furnished.

It may be mentioned here, however, that, in the case of some micro-organisms, several enzymes are often involved in the total proteolysis exhibited. In this case, the optimum  $pH$  (as determined by a measurement of the proteolytic products) represents the  $pH$  zone within which the sum of the products of the different enzymes is greatest. This need not be the optimum ( $H^+$ ) for any one of the enzymes involved. Its position will depend upon the relative production of the different enzymes. (Compare Demby's work on autolysis).

Wyeth (1918, 1919) has studied the effect of different  $H$ -concentration upon *B. coli*. In his report, however, he neglects entirely the alkaline fermentation of salts and ascribes all of the alkaline changes in  $pH$  to the formation of proteolytic products.



## V. Influence of Other Chemical Substances.

### 1. Salts of the metals --- general agreement with the E. M. F. series.

The presence of the salts of certain metals exerts a decided influence upon the life processes of the lactic acid bacteria.

Richet (1892) found that, while a very low concentration of these substances has a stimulating effect, slightly higher concentrations will inhibit their life processes, as evidenced by inhibition of fermentation; a still higher concentration is required to kill the cells. Later, Chassevant and Richet (1893) made a more extensive investigation of the influence exerted upon lactic acid bacteria by the chlorides of the metals.

They determined the amount required to prohibit the fermentation when a small inoculum is used (dose antigenetic) and the amount required when about 1000 times as large an inoculum is introduced ("dose antibiotique").

The authors considered these "doses" as the amounts required to stop multiplication, and that necessary to arrest the fermentation. Weigmann (1910) interprets them as the amounts required to inhibit fermentation and to kill the cells, respectively. More properly, perhaps, the first amount should be considered as that prohibiting further development and reproduction (the bacteria introduced with the inoculum eventually dying); the second dose probably represents the amount required not only to kill the cells but also to inhibit the action of any enzyme that might be liberated from the cells. (The amount of enzyme liberated from cells of the small inoculum would not produce a detectable amount of acid).

The following table is from their results.

The figures represent the gram molecules per liter required for their "dose antibiotique" or killing of cells and inhibition of enzyme action, and for their "dose antigenetic" or inhibition of growth. A study of this table shows a close relation between the influence of metallic ions and the position of the metal in the electromotive series.

Salt	"Dose anti-genetic"	"Dose anti-biotique"	Relative Harmful Effect	Remarks
LiCl	0.25	0.5	14	
BaCl <sub>2</sub>	0.125	0.25	13	{ In agree- ment with observations made in add- ing CaCO <sub>3</sub> and BaCO <sub>3</sub> as neu- tralizing substances.
SrCl <sub>2</sub>	0.125	0.25	13	
CaCl <sub>2</sub>	0.15	0.4	12	
MgCl <sub>2</sub>	0.5	1.5	15	
Al <sub>2</sub> Cl <sub>2</sub>	0.026	0.037	11	
MnCl <sub>2</sub>	0.0064	0.0085	10	
ZnCl <sub>2</sub>	0.0025	0.0035	8	Compare Beierjinke
CdCl <sub>2</sub>	0.00085	0.0021	6	
Fe <sub>2</sub> Cl <sub>6</sub>	0.004	0.005	9	
CoCl <sub>2</sub>	0.000065	0.00065	1	
NiCl <sub>2</sub>	0.000125	0.0002	5	
Pb(NO <sub>3</sub> ) <sub>2</sub>	0.0036	0.0061	9*	
H				
CuCl <sub>2</sub>	0.0015	0.0015	7	
HgCl <sub>2</sub>	0.000185	0.000185	4	
PtCl <sub>4</sub>	0.00025	0.00075	5	
AuCl <sub>3</sub>	0.0008	0.000165	2	

The relative effects of most of these metallic ions concur with the phenomena observed in the

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\* NO<sub>3</sub><sup>neg</sup> Cl

relation of toxicity of cations in general physiology. With most organisms the toxic effect of metallic ions varies inversely with the electrolytic solution tension of the metal.

Zinc, calcium, nickel, and especially cobalt are out of position, but these ions have been found in experiments on other organisms to be more toxic than explained by their position in the E. M. F. series. In view of the complexity of the medium used, these results cannot be interpreted as the absolute and specific effect of the ion. The agreement with the solution tension series might be even closer if the possible interfering action of the medium were removed. (Compare McClendon).

## 2. Ions exerting selective action on lactic acid bacteria.

### a. Zinc ion.

Zinc salts seem to exert a particularly harmful influence upon lactic acid bacteria.

M. W. Beijerinck claims that lactic micro-organisms are inhibited by so much lower concentrations of zinc salts than are acetic bacteria that the two groups may be distinguished by that characteristic.

### b. Fluoride ion.

Hydrofluoric acid and fluorides also seem to be particularly harmful to lactic acid bacteria.

Effront has applied this phenomenon in the fermentation industries. By the introduction of small concentrations of these substances to the medium of alcoholic fermentation, growth of lactic acid bacteria is prevented without disturbing the activity of the yeast. Effront (1894), however, has found that lactic acid bacteria may become accustomed to the presence of fluorides.

### c. Phosphates.

Euler and Svanberg (1917) report favorable influence upon lactic acid fermentation is exhibited by sodium phosphate. Although the authors claim that this is not entirely due to the buffer action of the phosphate, Van Dam does not believe their results justify the conclusion that phosphates exert a specific favorable influence upon the fermentation process itself.

The well known favorable effect of the addition of phosphates to increase the buffer value of media is evident in the growth of all micro-organisms. Although it is very advantageous in the case of lactic acid bacteria, it is just as necessary in the case of any micro-organism which produces substances tending to change the reaction from the optimum. Their effect, then, is not a specific action.

### 3. Common<sup>chemical</sup> disinfectants.

The lactic acid bacteria, being non spore bearers, are not relatively resistant to the common disinfectants. Even in milk, which is a protective medium, large concentrations are not required to inhibit, or at least retard, their development. A detailed consideration of the action of antiseptics upon lactic acid bacteria is not pertinent to the subject of this paper, as the addition of such substances to the media of agricultural lactic acid fermentations

is usually undesirable.

Thompson (1896) and Duclaux (1901) report the action of various disinfectants upon lactic acid bacteria.

Chloroform and benzene have been found to be not very toxic to lactic acid bacteria. In small doses, (as in case of metallic salts), formaldehyde has been found to exercise a stimulation of lactic acid fermentation. (Oppenheimer, 1913).

#### 4. Lecithin.

Epstein and Olsam (1912) have investigated the influence of lecithin upon lactic acid bacteria. They found that this phosphorized fat tended to increase the acid production of the lactic acid bacteria tested; it had an irregular effect upon gas production of lactic acid bacteria of the first group, varying with the sugar substrate and the species.

#### 5. Carbon dioxide and other gases arising in putrefaction.

Trillat (1912) found that carbon dioxide and possibly also other gases evolved in putrefaction tended to promote lactic acid production by lactic acid bacteria. The influence of these substances upon the reaction of the medium may also be a factor in these cases.

### VI. Influence of Different Concentrations of Salt.

As intimated under "Osmotic Pressure", high concentrations of salt and other sodium and potassium salts exert a certain influence upon all micro-organisms. This influence usually involves a



number of both physical and chemical factors. Due to the fact that salt is the substance usually employed to bring these factors into play in the environment of lactic organisms, the subject is discussed as a whole under the head of "Salt".

1. Influences operating ---physical and chemical.

The effect of different concentrations of salt upon lactic acid bacteria may be conveniently grouped under the chemical effect of the ions themselves and the physical and chemical effects induced in the environment by the presence of these ions. The chemical effect of the ions would include the effect of both cation and anion; this effect might be either specific or synergetic in presence of other ions. Apparently the chemical effect of the Na and Cl ions is slight. The reports (Rahn, 1917) of less retardation of growth by potassium salts than by sodium salts suggest that micro-organisms may not be absolutely indifferent to their presence.\* Some investigators (Aderhold, 1910) report favorable effect of relatively low concentrations of these ions upon lactic acid fermentation, but the complexity of factors involved precludes assigning this to chemical properties of salt.

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\* This fact may, however, be due to an activating influence of potassium ions.

The physical effects would include changes in osmotic pressure and freezing point of the solution.

However, the effect of concentrated salt solutions can rarely be analysed to one of these factors alone; it is usually a collective effect involving the effect of all of these factors both on the lactics and on their environment. Moreover, all of the life processes may not respond in the same manner to this collective effect. Again, liberated enzymes may not be influenced to the same extent as are the living lactics themselves.

## 2. Effect upon other conditions in the system.

The presence of certain concentrations of Na and Cl ions often exerts a contributing or counteracting influence on the effect of other environmental conditions confronting lactic acid bacteria. Low temperature effect is probably different in concentrated solutions of low freezing point. At ordinary temperatures, food conditions are likewise altered by presence of the ions, as seen in sugar content of sauerkraut brines.

Possibly the most important influence is the result of the effect of all these factors upon the microbial balance.

## 3. Effect upon microbial balance.

The collective effect of high concentrations of Na and Cl ions produces certain environmental

conditions in the medium. The various micro-organisms initially present differ in their fitness for this environment and it is this fitness which determines the microbial supremacy. Among these, the lactics, as a group, exhibit at least moderate resistance to media of high salt content.

4. Relation of different lactic acid bacteria to various concentrations of salt.

That at least some lactics possess a resistance considerably above many other micro-organisms is shown by reports of the following investigations. (A comparison of the salt concentrations reported below with the molar concentrations resulting shows that many lactics are quite resistant to osmotic pressure.)

Aderhold (1899) reports as follows the production of lactic acid in beans preserved in varying concentrations of brine:

6% NaCl,	1.593% lactic acid;
12% NaCl,	1.224% lactic acid;
25% NaCl,	0.216% lactic acid.

He found *M. pyogenes*, of our fourth group of lactic acid bacteria, and an organism apparently identical with *Strep. lacticus* to be the principal inhabitants of the above brines. Such resistance, however, is unusual and it is extremely doubtful if usual lactics can grow in solutions much above 12%. (Aderhold's method of calculating the concentration of the salt is open to question, which may explain this apparent great tolerance of high concentrations.)

Acid production may be due to other causes than life processes of lactic acid bacteria and it is better to judge the influence of NaCl upon the basis of rate of growth and inhibition of life. Aderhold (1899) investigated the effect of different concentrations of salt upon rate of growth of *B. coli* and *Bact. guntheri* (*Strep. lacticus*). He found the colon organism much less resistant than the common true lactic. The former

exhibited indifference to concentrations up to 2% and slow growth in higher concentrations up to 5%, beyond which no growth took place. The true lactic, however, grew in concentrations up to 3% as well as in the control; 5%, 6% and 8% allowed slow but distinct growth with weaker acid production.

In investigations of the influence of salt on the butter flora, Giltner and Baker found streptococci much more sensitive to salt than staphylococci and micrococci. They report exceptional resistance of some micrococci, many of which probably belong to our fourth group of lactic acid bacteria.

Evans (1918b) found that 10% NaCl in milk produced a lethal effect upon one strain of *Strep. lacticus* in five days; a hardier strain resisted the same concentration for ten days. She found *Bact. bulgaricum* much more resistant to high concentrations of salt than were her strains of *Strep. lacticus*.

Jensen (1919) reports the behavior of a number of strains of different lactic acid bacteria in varying concentrations of salt. He measured their tolerance by the amount of acid produced in 2% glucose broth, containing 0.5%, 2.5%, 5.5%, 10.5% and 15.5% NaCl. He found that very few were effected by 2.5% and that some seemed to grow even better than in the presence of 0.5%. Concentrations up to 5% (or approximately 0.9 m) were more or less harmful to all; 10.5% salt (approximately 1.8 m) stopped the growth of most of them.

Thus, it is seen that, while lactics are more resistant to the environmental conditions induced by high salt concentrations than many micro-organisms, salt beyond a certain concentration precludes their development and the various influences in such systems may even exert a disinfectant action. *Torulae* and certain halophilic bacteria survive in media of much higher concentrations and in such environments would dominate the system. Changes in environment produced by high salt concentrations and the consequent determination of microbial supremacy has a direct bearing in



many agricultural lactic acid fermentations.

5. Significance in agricultural lactic acid fermentation.

The salt content of salted butter is one of the factors determining microbial group ascendancy in this medium during its storage period. If held at a low temperature, micro-organisms decrease in numbers during storage. In many cases the salt concentration is among the factors involved in this inhibition of life. The resistance of the lactics, under the conditions prevailing in salted butter, surpasses that of the usual molds of butter, (Thom and Shaw, 1915), and that of many bacteria; other micro-organisms, especially liquefying torulae, (Brown, 1912), and some peptonizing bacteria, (Rogers, 1904), are more persistent than the lactic acid bacteria. Fetti-  
tick also reports that too high concentrations of salt suppress lactic acid bacteria and give control of the butter to undesirable micro-organisms. Further data on the rôle of salt at very low temperatures have been given in the discussion of the influence of different temperatures upon lactic acid bacteria.

In curing of certain cheeses salt concentration is again an important factor in the determination of microbial group supremacy. This may



extend to a choice between lactics as shown by Evans (1918b) in case of lactobacilli supplanting *Strep. lacticus* during the ripening of Roquefort cheese.

In sauerkraut and other pickled foods, the predominating type of micro-organism is largely due to the salt content of the brine. Here, a large part of the rôle of salt concentration is due to its influence upon the diffusion of sugars from plant cells into the brine. This results in a medium well suited for growth of lactic acid bacteria and the increased hydrogen ion concentration resulting from lactic growth exerts its usual inhibitive effect upon many undesirable micro-organisms.

This factor was recognized by Aderhold (1899) in one of the first investigations of this phase of lactic acid fermentation. He advised the addition of small amounts of dextrose to the brine of gurbens to preclude the possible gain of ascendancy by undesirable organisms during the time before the diffusion from the plant cells produced brine of sufficient sugar concentration to favor lactic acid fermentation.

# INFLUENCE OF ENVIRONMENT UPON LACTIC ACID BACTERIA

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#### IV. Unfavorable Associative Action Exerted by Lactic Acid Bacteria.

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#### V. Other Examples of Associative Action in Lactic Acid Fermentation.

## INFLUENCE OF ENVIRONMENT UPON LACTIC ACID BACTERIA

### C. INTERMICROBIAL INFLUENCES

#### I. General Discussion of Mutual Relations Between Micro-organisms.

##### 1. Significance.

Since it is "pertinent to consider micro-organisms in their natural surroundings as significant as in a laboratory pure culture", (Marshall, 1915), a consideration of the biology of the lactic acid bacteria would be incomplete without a discussion of the biological relations of the lactics to other micro-organisms which are almost always present with them in the media of natural lactic acid fermentations. "Nature prepares but few pure cultures and in any natural microbial process there are concerned, at least in the beginning, always more than one species". Probably nowhere is this more true than in agricultural lactic acid fermentations.

##### 2. Assumed cases of no associative influence.

If, in these mixed cultures, each grew as if alone, the change produced in the medium would be merely the resultant of all the physical and chemical changes brought about by the life processes of the different micro-organisms present. This relation, however, seldom exists.

### 3. Cases in which type gains ascendancy.

In many cases, one species or group of micro-organisms, which is best adapted to the medium, becomes more or less dominant and, at times, gains the ascendancy to such an extent that the ultimate change in the medium is practically the same as that which would occur if it alone were present. (Examples of this relation are often seen in the early stages of the natural fermentation of milk. In such cases, mutual influence does not enter; it is merely a question of which micro-organism survives in the struggle for existence. Whether or not it is the lactic acid bacteria which gain the ascendancy will determine whether lactic acid fermentation will be the change induced in the medium. The gaining of ascendancy in the medium by the lactic acid bacteria will depend, in such cases, upon the relative fitness of the environment for the different micro-organisms of the flora (the fitness of the environment will be decided by the temperature, oxygen, food,  $(H^+)$ , and other relations discussed in preceding topics), and upon the relative numbers of the lactic acid bacteria and the other organisms initially present in the system.

### 4. Cases of evident associative influence.

In other cases, certain micro-organisms exert a decided influence on the others present, per-



haps favoring them in their development and their physiological functioning, perhaps hindering them. As this mutual influence or associative action of micro-organisms assumes great importance in many agricultural lactic acid fermentations, several investigators have studied the biologic relations existing between lactic acid bacteria and micro-organisms associated with them.

## II. Marshall's Explanation of Associative Action in Lactic Acid Fermentation.

The first extensive investigations in the field of associative action in lactic acid fermentation were concerned with cases in which the life processes of lactic acid bacteria were furthered by other micro-organisms associated with them. In the report of these investigations, Marshall (1903, 1904, 1905) enumerates some of the explanations offered as possible for the furthering of lactic acid fermentation by the associate micro-organisms.

### 1. Desirable change produced in medium.

#### a. Products formed by the associate bacteria, which may,

(1) Provide a better pabulum for the lactics.

(2) Neutralize the acid formed by the lactics, and thus stimulate their growth.

### 2. Changes in environmental factors.

Associate bacteria may exert some influence upon the lactic acid bacteria in their relation to oxygen supply and other environmental conditions.

### 3. Production of acid by the associate.

Acid produced by the associate micro-organism may account for the increased acidity in the combination; (in which cases, there may be no associative influence proper).

These explanations still hold and the subject is well approached by a consideration of the evidence in favor of each of them.

### III. Examples of Favorable Associative Influence Exerted upon Lactic Acid Bacteria.

#### 1. Desirable change in medium of growth.

##### a. Better pabulum.

Marshall found that lactic acid bacteria grown in milk in which peptonizing bacteria had grown produced a larger amount of lactic acid than when grown in milk in pure culture. It will be recalled that "peptone" is a very favorable nitrogen medium for lactic acid bacteria and that the content of undigested milk is relatively low. Furthermore, many lactic acid bacteria exhibit very slight ability to attack casein, while the associate bacteria possess evident power of hydrolyzing this protein to simpler products more easily utilized by the lactic.

This interpretation of Marshall was strengthened by the results later obtained by Jensen in milk cultures in which the casein had been hydro-

lyzed without the aid of associate micro-organisms.  
(See "Influence of Nitrogenous Food").

These facts indicate that, in some cases at least, the furthering of the life processes of the lactic acid bacteria is due to an alteration of the casein to products offering a more available source of nitrogen to the lactics;\* moreover, that this favorable influence has been due to the life processes of the associate micro-organisms.

The stimulation of the lactics occurred in many of these experiments when the associate bacteria were removed by sterilization of the milk before being inoculated with the lactic acid bacteria. It is safe to assume that in these cases the associative influence is due to the better pabulum for the lactic acid bacteria, which is presented by the medium after its enrichment with thermolabile products (probably hydrolytic products of casein) of the associate.

Koestler (1907) explains the favorable influence of *B. mesentericus* upon lactic acid bacteria as due partly to the change this associate bacterium produced in the nitrogenous nourishment offered to the lactics. Northrup (1912) also ascribes part of the favorable associative influence of her red yeasts to a similar action on the casein.

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\* Kendall's (1910) observation of associative action in case of *B. coli* and *B. mesentericus*, when grown in milk, furnishes another example of conditions similar to those first observed by Marshall. Here, too, the proteolytic action of the actively peptonizing bacillus would furnish a better pabulum for the lactic acid bacteria. The tendency of this action to regulate the ( $H^+$ ) of the medium would still further enhance the continual growth of the colon bacillus.

Besides these examples of favorable associative action due to a recognized change in the nitrogenous material offered to the lactics, all cases are not so easily explained. In some of Marshall's experiments the stimulating substance produced by the associate bacteria proved to be thermolabile. The favorable influence upon the lactics in these cases must have been due to some unrecognized thermolabile product or to the action of enzymes or toxin-like substances secreted by the associate micro-organism, or possibly comparable to Northrup's "other products". (See below.)

b. Favorable change in ( $H^+$ ) of the medium.

The favorable influence which an associate organism would exert upon the lactics by neutralizing the acid produced is quite evident from the discussed relation of ( $H^+$ ) to lactic acid bacteria. In this phase of favorable associative action, the associate micro-organism may play the role of ( $H^+$ ) regulator, either by using up the lactic acid directly or by the secretion or excretion of products which will exert a neutralizing or buffer action upon the acidity produced by the lactic acid bacteria.

(1) Lowering of ( $H^+$ ) by direct combustion of lactic acid.

The lactic acid consuming power of *Oidium lactis* is well known; it is known also that it produces products from nitrogenous substances which would tend

to neutralize the lactic acid formed in associated cultures. From this standpoint is explained the common laboratory observation that lactic acid bacteria, pure cultures of which die rather soon in milk, live for a long time in milk cultures in which *Oidium lactis* is also present.

Troili and Peterson (1899) report a case of this kind in which the length of life of lactic acid bacteria was increased two and one half months in the presence of this mold. In her investigation on associate action of red yeasts upon lactic acid bacteria, Northrup (1912) believed part of the furthering of the life processes of the lactics was due to the direct combustion of the lactic acid by the yeast.

(2) Formation of products having  
a neutralizing or buffer  
action.

Besides these examples of lowering of the ( $H^+$ ) by actual combustion of lactic acid, many times this effect is brought about by neutralizing or buffer substances introduced into the medium by the life processes of associate organisms. The production of ammonia and alkaline fermentation of organic acid salts would be among the means by which associate bacteria could lower the ( $H^+$ ). Moreover, the investigations of Von Dam (1918) and others show that the hydrolysis of proteins by peptonizing bacteria would increase the buffer value of the medium by formation of substances capable of resisting changes in ( $H^+$ ).



## 2. Changes in oxygen concentration or other environmental factors.

In some lactic acid fermentations the favorable associative action may be due to an influence exerted by the associate micro-organism upon the oxygen supply and other conditions in the environment of the lactic acid bacteria. Since the time of Pasteur it has been known that the presence of obligate aerobes favors the growth of anaerobes. It has also been shown that many lactic acid bacteria grow best in low oxygen concentration. Hence, it is but natural that the presence of obligate aerobes with the consequent lowering of the oxygen concentration should facilitate the growth of lactic acid bacteria.

Koestler interpreted the favorable influence of his associated obligate aerobes to such a lowering of the oxygen concentration to a zone more nearly the optimum for the lactics. Northrup's (1912) red yeasts were also obligate aerobes and she believes this accounts for one of the phases of their beneficial associative action. (Beierjink has used yeasts in association for the culture of anaerobes).

This factor might be particularly potent in cases in which the associated aerobe formed a pellicle on the surface, thus not only using up the oxygen in the liquid but also mechanically hindering its entrance. This was the case in one of the experiments of Koestler, who grew a mycoderma in association with *B. casei*.

Doubtless, at times, more than one of the factors included in these explanations are required to account for all phases of the beneficial associative action.

### 3. Possible production of acid by the associate.

The possibility of production of acid by the associate micro-organism may at times account for the apparently favorable influence upon lactic acid bacteria. The change induced in the medium in such cases would be merely the resultant of the forces exerted by the life processes of the different organisms present. These cases are not, properly speaking, examples of associative action. Moreover, probably the type which had the greatest toleration to ( $H^+$ ) would produce as great an amount of acid growing alone as it would in the presence of another type which was less resistant to acids (provided both types produced the same acid).

### IV. Unfavorable Associative Influence Exerted by Lactic Acid Bacteria.

#### 1. Significance in agricultural lactic acid fermentation.

Biological relations between lactic acid bacteria and other micro-organisms are not limited to cases in which the associative influence is beneficial. Many times the lactic acid bacteria gain ascendancy in media in which other organisms are present. This may give rise to conditions analogous to those proposed by Marshall with the exception that the change produced will have an inhibitory influence upon the other micro-organisms present.

## 2. Examples of such associative action.

### a. Influence of $(H^+)$ .

We know that high  $(H^+)$  will inhibit the growth of many bacteria, and more, that prolonged presence in such a medium may have a lethal effect. The high  $(H^+)$  brought about by the lactic acid produced by the life processes of lactic acid bacteria often results in the complete inhibition of other micro-organisms present, some of which, if not inhibited, would produce undesirable changes in the medium.

Striking examples of this phenomenon are seen in many agricultural lactic acid fermentations, such as in the natural lactic acid fermentation of milk, the preparation of sauerkraut, etc..

### b. "Other products."

Northrup (1911) has shown that in some cases the acid itself may not be the only factor to account for the unfavorable influence of the products of lactic acid bacteria upon other microbes.

In her interesting investigation on the influence of products of growth of lactic bacteria upon *B. typhosus*, she observed that the germicidal action of cultures of different lactic acid bacteria was not directly proportional to the degree of acidity of each. (Her measurements are not, however, in terms of  $(H^+)$ ).

It is possible that at least some lactic organisms produce "other products" than acids, which are also a factor, as well as the actual  $(H^+)$ , in the inhibition of growth of other micro-organisms associated with them.

Jensen (1904) reports that *B. lactis acidii* exerts an unfavorable associative influence upon *M. casei liquefaciens* when grown in milk cultures. The micrococci disappear, even when calcium carbonate is present in the milk. (Other factors may be involved here. Moreover, the presence of calcium carbonate does not insure neutrality throughout the medium. Compare Clark (1920) ).

These unknown products have also been recognized by other workers, in the case of micro-organisms closely related to the most common lactic acid bacteria.

The following reports may not be strictly within the boundaries of associative influence, as they are based upon the effect of such products upon the ability of certain micro-organisms to initiate growth. The initiation of growth (especially of small inocula) may exhibit decidedly different relations than would the actual associative growth of the same organisms in more equal proportions, (in the latter case, differences in rate may be the deciding factor). It is probable, however, that products inhibiting initial growth would exert at least a quantitative effect upon associative growth, even though present in smaller concentration.

After apparently incomplete investigations, Marmorck (1902) announced that filtrates of bouillon cultures of streptococci of human origin would no longer serve as media for the growth of other streptococci of human origin. Marmorck regarded this reaction as highly specific and indicative of "*l'unité des streptocoques pathogènes pour l'homme.*" This test has, however, been proven to be non-specific.

J. H. Brown (1919) has reported relations between certain pneumococci and *B. coli*, which seem to be explained only by the influence of unknown metabolic products. He found that pneumococci may fail to grow in plain bouillon, which has been "metabolized" by *B. coli*. The inhibitory influence of the products of growth of the colon organism was likewise exhibited even after the reaction had been adjusted and a fermentable sugar added to the medium. Brown believes that "apparently the ability of a streptococcus or any other organism to grow in a medium metabolized by another organism is dependent wholly upon the ability of the former to tolerate the metabolic products of the latter or (and) to utilize certain nutritive substances not utilized by



the latter." He emphasizes the fact that such relations are not wholly quantitative and that "a relatively poor growing organism may produce certain substances which even in small quantities may inhibit the growth of organisms of greater but different metabolic activity."

The influence of such products upon the end point of lactic acid fermentation will be discussed under "Theoretical Progress of Lactic Acid Fermentation".

#### V. Other Examples of Associative Action in Lactic Acid Fermentation.

The literature furnishes many other references to associative action as a factor to be considered in a discussion of the relation of lactic acid bacteria to their environment.

Evans, Hastings and Hart (1914) found associative action a factor in the ripening of cheese. They also report its influence on sugar fermentations: "In many cases acid was produced from a given substance by the associated action, when neither culture working alone would give such a reaction." Quantitative as well as qualitative differences were exhibited by different associates.

Hammer (1914) reports the influence of the presence of lactic organisms upon the color production of *B. cyanogenes*.

Buchanan and Hammer (1915) believed associative action of certain organisms to be one of the factors involved in the development of slimy and ropy milk in the presence of lactic streptococci.

Hammer (1919) has also reported that the relative production of volatile acids in "starters" seems to depend upon the associative action of the lactic streptococci and other micro-organisms.

Smith and Smith (1920) have shown that certain types of paratyphoid bacilli exert an inhibitory influence upon gas production by *B. coli*. Their work is especially interesting in that it suggests that associative influence may exert a selective action upon certain functions of micro-organisms or upon certain of their enzymes. They found that the above inhibitory in-



fluence did not affect the production of acid.

Barthel and Sandberg (1919) found that no associative action is exhibited by *Strep. lacticus* in conjunction with lactobacilli in so far as proteolysis of casein is concerned.

Luxwolda (1911) reports other examples of associative influence in lactic acid fermentation.

In a study of cheese ripening, Weigmann (1899) investigated the associative effect of introducing inocula of different sizes of different organisms into sterile milk.

Rosengren (1912) ascribed the "yeasty" taste of butter to the associative action of lactic acid bacteria and yeasts.

Gayon and Dubourg (1904) reported that their lactic acid bacteria exerted an unfavorable associative influence upon alcohol production by yeasts. Müller-Thurgau and Osterwalder (1912) did not find so pronounced an associative influence between yeasts and lactic acid forming micrococci from wine.

Gratz (1912) has reported on the associative relations between lactobacilli and the colon-aerogenes group.

Bonska (1903) investigated the associative effect of lactic acid bacteria and bacilli of the *B. subtilis* group in cultures held at different temperatures.

Among the first reports on association in lactic acid fermentation were presented in the earliest studies on fermented milk drinks. An

associative action between yeasts and lactobacilli was suggested in the pioneer reports of Bern, Kranhals and others (before the use of the plate method); was recognized by Beijerinck (1869); and was claimed to be demonstrated in the case of a streptococcus by Freudenberg (1897). He stated that a strain of yeast, pure cultures of which did not attack lactose, fermented lactose of mixed with a streptococcus from Kefer. This associative influence Freudenberg ascribes to a hydrolysis of the lactose by the (assumed) lactase of the streptococcus.

This reason, commonly given for the associative phenomena in fermented milks, would seem to require the presence of an extracellular lactase. Direct evidence of the possession of such an enzyme by streptococci has never been made and (from analogy with the pneumococcus) it seems probable that the lactase of streptococci would be endocellular. Associative influence of lactobacilli upon yeasts in fermented milks has also been ascribed to the preparation of the lactase for the yeasts by the lactase of the bacilli. This is also questionable for it is probable that the endocellular lactase possessed by some yeasts escaped the observation of the earlier workers; moreover, the lactase of lactobacilli has usually been found to be endocellular.

Marshall (1915) gives an extensive treatment of "Microbial Associations", including the history of the subject in general biology. See also Duclaux (IV, pp. 738-747).

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F. PHYSIOLOGICAL EFFICIENCY OR FERMENTING  
CAPACITY OF LACTIC ACID BACTERIA.

PHYSIOLOGICAL EFFICIENCY OR "FERMENTING CAPACITY" OF  
LACTIC ACID BACTERIA

- I. Definition of Term.
- II. Interpretation of "Fermenting Capacity".
- III. Fermenting Capacity of Different Lactic Acid Bacteria.
- IV. Dependence of Fermenting Capacity upon Other Conditions.
  1. Influence of age of cells.
  2. Influence of fitness of the environment.
- V. Decrease in Fermenting Capacity or Degeneration of the Lactic Acid Bacteria.

## PHYSIOLOGICAL EFFICIENCY OR "FERMENTING CAPACITY OF LACTIC ACID BACTERIA

This topic is confined to a consideration of the "true" lactic acid bacteria (second and third groups). This is done to avoid complications arising from a consideration of complex products of the acid gas lactic acid fermentation. The same principles will apply to some extent to members of the other groups.

### I. Definition of Term.

It has been mentioned earlier in this paper that some lactic acid bacteria induce lactic acid fermentations in which greater amounts of lactic acid are formed than in the fermentations brought about by others. Different terms have been proposed to designate the relative ability of different bacteria to produce lactic acid. Among these, perhaps the term "fermenting capacity", suggested by Rahm, is the most appropriate.

### II. Interpretation of "Fermenting Capacity".

This "fermenting capacity" is interpreted upon different bases by different authorities. By most the "fermenting capacity" is considered as a rate --- the rate of production of lactic acid by certain lactic acid bacteria. To do this, it is necessary to eliminate other variables in order that a constant may be presented on which to calculate the amount of lactic acid as a rate --- i.e., as the amount of lactic acid produced in a given time.



It is obvious that the number of lactic acid bacteria present would be one of the factors in the amount of lactic acid produced during a given time, and that the rates of lactic acid production by different lactic acid bacteria would be comparable only if calculated upon the basis of a given number or amount of lactic acid bacteria. By many investigators this fact has been ignored and for this reason their measure of the "fermenting capacity" has been a measure of the lactic acid resulting from two factors, the multiplication of the cells, as well as the "fermenting capacity" of a given number or mass of lactic acid bacteria.

### III. "Fermenting Capacity" of Different Lactic Acid Bacteria.

Duclaux tries to eliminate this first factor by introducing such a large inoculum that no further increase in number of cells can take place. He then bases the "fermenting capacity" upon the amount of lactic acid produced by a given weight of lactic acid bacteria.

Rahn attacks the question from a somewhat different angle and investigates the "fermenting capacity" of a single cell. He considers the "fermenting capacity" of a single cell to be a measure of the amount of enzyme in the cell. (Compare relation of concentration of enzyme to velocity of reaction, discussed under "Theoretical Progress of Lactic Acid Fermentation".)

By this method of approach he shows that the "fermenting capacity" of a single cell varies with the strain. He found the weakest strain of *Strep. lacticus* possessed a "fermenting capacity" of  $(17.4 \times 10^{-10})$  mg. per hour, while the strongest strain produced  $(32.5 \times 10^{-10})$  mg. of lactic acid per hour; average "fermenting capacity" of various strains was  $18 \times 10^{-10}$  mg.. This shows that one cell forms about its own weight of lactic acid in one hour, which in turn requires that a cell ferment its own weight of sugar in that time.

This large amount of food used is in agreement with the relatively small energy yield of the chemical reaction of lactic acid fermentation as compared to those of processes of complete oxidation (see "Energy Relations"). Moreover, in most cases, the lactic acid fermentation of the sugar is the only, or at least the principal, exothermic reaction occurring within the cell. These two facts --- (1) the small energy yield per gram sugar; (2) the dependence upon this relatively unproductive reaction for most of their energy --- account for the relatively large food requirements of lactic acid bacteria.

Working with lactic acid bacteria of the third group, and using different methods, Wehmer calculated the amount of lactic acid formed per day by a given weight of the cells. As it is well known that the lactobacilli grow and ferment slowly, his result is well in accord with the more accurate determinations of Rahn. This furnishes an example of an organism which has a slow "rate" of lactic acid production, although its final potential acid production is high.

The "fermenting capacity" is a rate and must not be confused with final ultimate acidity reached. This latter is dependent upon other properties, especially the ( $H^+$ ) toleration of the organism. These points will be discussed later. (See "End Point of Lactic Acid Fermentation").

The distinction between rate of lactic acid production, (of which the fermenting capacity is an expression), and final amount of acid production is plainly seen in a curve of lactic acid fermentation. The fermenting capacity would be a function of the slope of the curve; the final amount of lactic acid, the highest ordinate. (Compare curves shown in the following division of this paper.)

It is but natural to assume that different species possess different "fermenting capacities". Duclaux found considerable differences existing in the amounts of lactic acid produced by given masses of different species of lactic acid bacteria. Besides this difference between species, the different strains of lactic acid bacteria of the second group exhibited very different "fermenting capacities" in Rahn's investigation.

#### IV. Dependence of "Fermenting Capacity" upon Other Conditions.

##### 1. Influence of age of cells.

Not only do the different strains possess different "fermenting capacities", but the same strain exhibits a variation in its "fermenting capacity", according to the age of the cells. Rahn found that the "fermenting capacity" of the cells was greatest during

the early periods of the life history of *B. lactis acidii*. (See also work of Rahn and of Grima reported in the following division.)

Recognition of such a period of greatest activity of the lactics, as well as a knowledge of the "fermenting capacity" of different strains of lactic acid bacteria, is of the greatest significance in the preparation and use of starters in the lactic acid fermentations of the dairy. (See "Theoretical Progress of Lactic Acid Fermentation".)

## 2. Fitness of the environment.

It must be remembered that the "fermenting capacity" of a strain is not an independent function of the cell and that the same strain would probably exhibit a different "fermenting capacity" when grown under different conditions. This property will vary with the biological relations discussed in preceding topics ---any change in the conditions which affects the physiology of the organisms will also affect the products of their life processes.

## V. Decrease in "Fermenting Capacity", or Degeneration of the Lactic Acid Bacteria.

Lactic acid bacteria respond to unfavorable conditions of the environment in the same way as do most organisms --- by a decrease in activity of their life processes. This extends not only to a decrease

in multiplication but also to a decrease in "fermenting capacity" of the cells actually present.

Among the unfavorable conditions in the environment, perhaps none is more important than the presence of an accumulation of metabolic products. (See "End Point of Lactic Acid Fermentation".) Upon this basis the degeneration of lactic acid bacteria in old cultures is best explained. Just as in systematic or descriptive bacteriology a culture must be invigorated and acclimatized before a correct expression of its biochemical activity can be obtained, so in lactic acid fermentation the lactic acid bacteria must be in vigorous, actively growing condition before their life processes can proceed to advantage.

The literature presents many references to a complete loss of fermenting ability by a once actively fermenting strain, due to age of the culture and to the addition of unfavorable substances to the medium. (Grotenfeld, Nencki, Schierbeck, Kruse, Weigmann, Rahn).

Reports of loss of power of the common lactic streptococcus to coagulate milk are not infrequent.

Such reports are, however, often due to the use of inocula from old and unrejuvenated cultures. Strains have been observed in this laboratory to fail to coagulate milk when inoculated from old cultures. These strains have, without exception, always gradually increased in activity by successive seeding. In all cases, these lactics have been able to coagulate milk readily after this treatment.



Many of the reports of degeneration of lactic acid bacteria are due to the improper care of stock cultures.

Media should be used which do not permit the attainment of high (H<sup>+</sup>)'s by the growth of the organism. Comparatively large inocula should be taken from the stock cultures in the first subtransfer.

Jensen claims that small inocula and the storage of cultures at too high temperatures account for many cases of "degeneration" of lactic acid bacteria. (Jensen, however, stored his cultures at 18° C.).

Variations in the ability of lactic acid bacteria to ferment different sugars is a much mooted question. Many of these cases are due to reasons discussed above.

For a review of variability of sugar fermentations, see the conclusions reached by Brown in his study of streptococci. He also furnishes a complete review of the literature on this question.

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G. THEORETICAL PROGRESS OF LACTIC ACID FERMENTATION.

## THEORETICAL PROGRESS OF LACTIC ACID FERMENTATION

### I. Introduction.

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2. Process Involved in the Metabolism of Lactic Acid Bacteria.

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### III. Representation of its Progress.

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3. Choice of basis for division.
  - a. Grimm's division into phases on changes in slope.
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  - c. Attempted explanations of above discrepancy.
    - (1) "Incubation stage" of lactic acid fermentation.
    - (2) Mechanism of lactic acid bacteria zymase.
    - (3) Incomplete data.
    - (4) Delay in multiplication.
      - (a) Discussion of the so-called "lag period".
      - (b) Significance in agricultural lactic acid fermentation.
  - d. Dismissal of this phase of the fermentation.



2. Phase of rapidly rising slope.

a. Conditioning factors.

(1) Concentrations of substrate and of enzyme.

(2) Activity of the catalyst.

b. Combination of first two phases into one period.

c. Practical significance of this period.

d. End of dominance of accelerating influences.

VII. Period of Retardation of Enzyme Action.

1. Phase of gradual inhibition.

a. Conditioning factors.

b. Concentration of substrate not the absolute factor.

c. Characteristics of this phase.

2. Last phase of the fermentation.

a. Actual inhibition of the reaction.

b. Practical significance.

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1. Determination of end point.

2. Factors involved.

a. Utilizable food.

b. Hydrogen ion concentration.

c. Other products of the fermentation --- as the lactate ion, molecular lactic acid and "other products" of unknown origin and nature.

d. Relative importance of the hydrogen ion factor.

e. Cumulative effect --- "Prohibitory concentration product".

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1. Influence of food upon rate of lactic acid production.
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X. Reversal of Reaction.

1. Reports of combustion of lactic acid itself.
2. Fermentation of salts of organic acids.
  - a. Simultaneous acid and alkaline fermentations.
  - b. Significance in agricultural lactic acid fermentation.
  - c. Significance in interpretation of presence of the colon-aerogenes group in various products.

## THEORETICAL PROGRESS OF LACTIC ACID FERMENTATION

### I. Introduction.

#### 1. Limitation of discussion.

The first part of this discussion is again limited to lactic acid fermentations induced by the "true" lactic acid bacteria, for much the same reasons as in the preceding division.

The preceding article on the biology of the lactic acid bacteria has shown the influence of the milieu upon the life processes of lactic micro-organisms and also the action of these organisms upon certain substances. An attempt to show the theoretical progress of a lactic acid fermentation consists, to a certain extent, in following the life history of the lactic acid bacteria in a certain medium. Here, the reactions induced by the life processes of the lactics will be influenced by the various environmental conditions confronting them. It will be necessary, therefore, to impose rather definite conditions in the discussion following.

#### 2. Process involved in the metabolism of lactic acid bacteria.

Let it be assumed that "true" lactic acid bacteria are inoculated into a medium well adapted to their needs and that other conditions in the environment are at the optimum. Much of at least the early history of their growth will be more or less parallel to that of any micro-organism. The lactic acid bac-

teria will attack the utilizable food presented to them in order to obtain energy for their life processes and substance for cell structure. In the metabolism of these organisms, the general principles of microbial physiology, as well as the more specific physiological characters of lactic acid bacteria discussed above, will determine the progress of their life history.

The fermentable sugar will diffuse through the cell wall and be attacked by the zymase within the cell of the lactic bacterium; the nitrogenous substances presented in the medium will be acted upon by other enzymes of the lactics. By means of these and other processes involved in their metabolism, the lactic acid bacteria obtain energy and utilizable food, both of which are required for growth and multiplication.

## II. Method of Measuring the Progress of Lactic Acid Fermentation.

The progress of a chemical reaction is usually determined by measurements of the concentration of its products. In the same way, a correct knowledge of the progress of lactic acid fermentation, essentially a chemical reaction, may best be obtained by accurate determinations of the products resulting from the life activities of the lactic acid bacteria. The principal product of "true" lactic acid fermentations is lactic acid. Hence, it is usually upon mea-

surements of the concentration of this substance that a determination of the progress of the fermentation is based. (Difficulties are often encountered, however, that seriously interfere with accurate determinations of lactic acid in the media of lactic acid fermentation.)

Rubner (1906), however, has investigated the progress of lactic acid fermentation by calorimetric determinations of the heat energy produced in lactic acid fermentation.

### III. Representation of the Progress of the Fermentation.

The progress of lactic acid fermentation is most clearly shown by plotting the concentrations of the reaction products which are present in the medium at different time intervals during the history of the fermentation. The acid produced is usually represented as degrees of acidity or weights of lactic acid which have been produced at a given time. (See Figure 1.)



Figure 1.

Representation of the Progress of a Lactic Acid Fermentation.  
"Fermentation Curves"

-- Data from Rahm (1911), Table V.

-- Curve given by Grimm.

Abscissae -- Acid produced at time  $t$ .

Ordinates -- Time.

The following comments on the character of the curves have special reference to the division of "Fermentation Curve" to be given later (p.     ).

Period I.

Concave upwards: acceleration.

Phase 1.

Zero or very gradual slope (for reasons given in discussion).

Phase 2.

Rapidly rising slope: acceleration.

This phase of the curve is dominated by the influence of increasing concentration of the enzyme. (Compare Fig. 3). It follows closely the curve of growth (compare Fig. 2).

Period II.

Concave upwards: retardation.

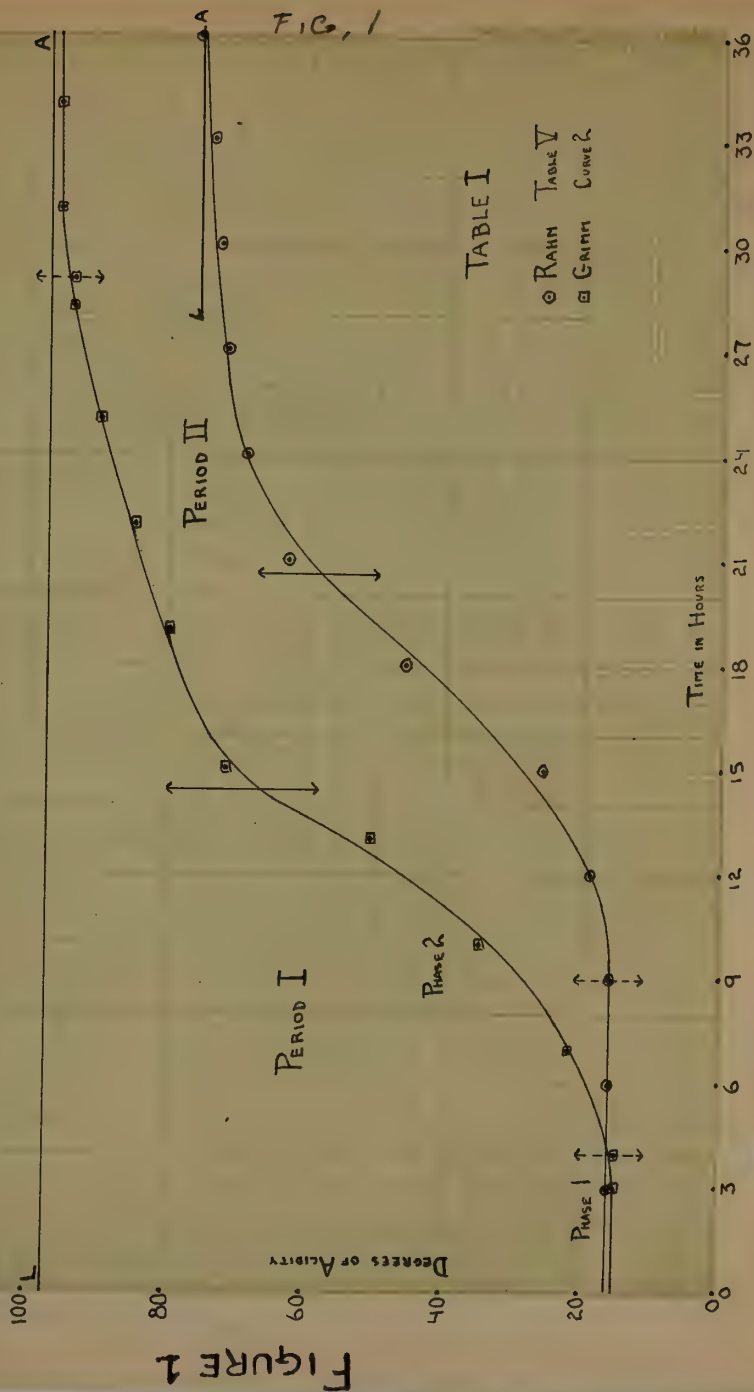
Phase 3.

Slope changes but slightly during this phase; no acceleration and no retardation. (See discussion for explanation).

Phase 4.

Slope rapidly decreases and finally becomes zero; retarding and inhibiting influences now dominant.

# "FERMENTATION CURVES": Representations of the Progress of Lactic Acid Fermentations.



#### IV. Interpretation of Progress of the Fermentation.

##### 1. Interpretation of charts showing progress of the reaction.

###### a. Reaction elements involved.

It is obvious that several forces are involved in the production of the reaction product whose concentration is plotted in the above figure. Its concentration is due to the catalytic action of the lactic acid bacteria zymase upon the fermentation sugar, but both of these "reagents" are changing in concentration during the reaction. Consequently, the course of the lactic acid fermentation process is the result of the, at times, simultaneous action of two forces --- multiplication of the lactics, and catalytic action of their lactic acid producing enzyme.

###### b. Composite "fermentation curve".

The curve, as a whole, then, is not a simple curve, but a composite curve of the action of the enzyme itself and of the curve of growth by which the lactic acid bacteria zymase increases in concentration. Rahn (1910) calls such composite curves "fermentation curves".

###### c. Significance of slope of the curves.

The slope of the curve of any reaction represents the speed of production of the reaction product whose concentration is plotted. The slope at any point in the above figures represents the speed of production of lactic acid at that time. The derivative

Figure 2.

Comparison of Curve of First Order Reactions with the  
Curve of Growth.

Exponential Curve (I)

Concave upwards throughout; slope always rising.

Curve of Growth

An exponential curve; slope always rising until certain factors, (which also cause decrease in the slope of the "Fermentation Curve" itself), impose their retarding influences upon growth.

Logarithmic Curve

Concave downwards; slope decreasing.

Curve of Monomolecular Reactions

A logarithmic curve; slope falling, due to decreasing concentration of the reacting substance.

"Fermentation Curve" as a Composite Curve of Elementary Exponential Curve of Growth and of Logarithmic Curve of First Order Reactions (Glucose — Lactic Acid).

See text. Also compare figures and observe the influence of the curve of growth upon the "Fermentation Curve" during the period of active growth of the culture.

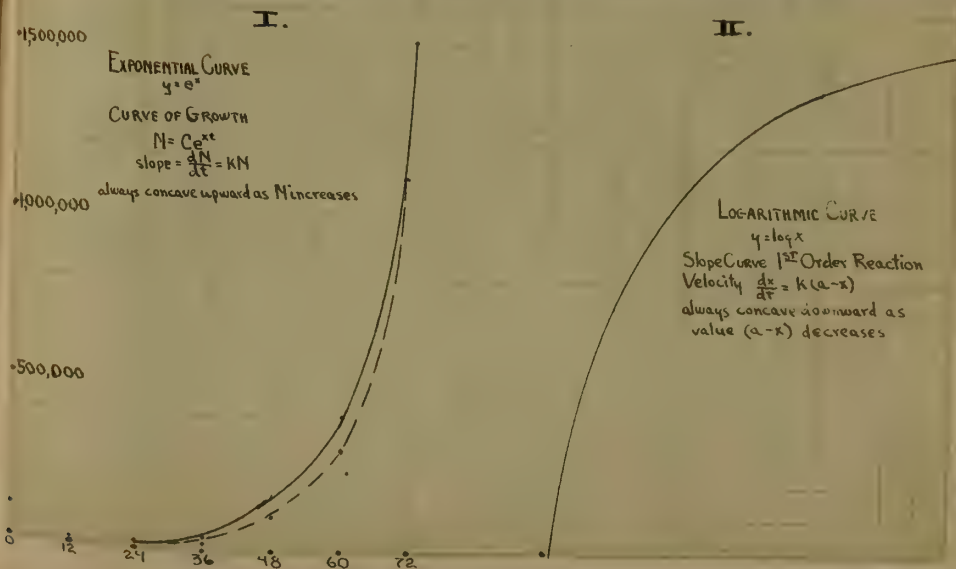
of the lactic acid produced in respect to time,  $\frac{dA}{dt}$ , is the rate or speed of lactic acid production.

The slope of the curve of lactic acid fermentations is indicative of the conditions determining the rate of lactic acid production and, consequently, of the progress of the fermentation. The significance of changes in the slope of the curve furnishes the basis for the division of the curve into periods given later in the discussion.

Figure 2.

COMPARISON OF THE CURVES OF FIRST ORDER REACTIONS WITH THE CURVE OF GROWTH.

- I. Exponential Curve (theoretical)      II. Logarithmic Curve;  
 — Curve of Growth      Curve of Monomolecular Reactions.





2. Relation of fermentation curves to curves of first order reactions.

a. Variables influencing the course of unimolecular reactions.

Plotting the course of ideal monomolecular reactions gives a logarithmic curve. (See Fig. 2). The general form of certain parts of "lactic acid fermentation curves" (Fig. 1) is in a general way similar to that of a unimolecular reaction. However, even though the lactic acid fermentation reaction itself is a monomolecular chemical reaction, the course of the fermentation cannot be considered to follow closely the law of Guldberg and Waage.

The course of a monomolecular chemical reaction, in conformity to the law of mass action, is dependent upon the concentration of the reaction substance. The velocity of catalytic unimolecular reactions, in the presence of a constant catalyst\*, is proportional to the concentration of the substance during the course of the reaction. Then, in monomolecular reactions, which can be expressed by logarithmic

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\*This is true, only if the catalyst is present in not too low concentration; see later in the paper.

curves, the independent variable is the concentration of the reacting substance, the velocity is decreasing throughout the reaction, and the ultimate zero slope of the curve, or equilibrium point of the reaction, is reached because of decreased concentration of the reacting substance.

b. Variables influencing the course of the lactic acid fermentation reaction.

(1) Relation to those of unimolecular reactions not absolute.

In spite of the more or less general similarity between certain parts of the fermentation curve and the logarithmic form of unimolecular reactions, the lactic acid fermentation curve is conditioned by other factors. Investigators of disinfection and hemolysis encounter similar obstacles in attempts to express the progress of these biological phenomena by the curves of unimolecular reactions.

(2) Variables determining course of the fermentation reaction.

The variables in the case of lactic acid fermentation cannot be limited merely to the concentration of the substrate, for here the change in concentration of the catalyst is a dominant factor. Hence, the relation of the concentrations of the enzyme and substrate to the velocity of the lactic acid fermentation reaction is different than those prevailing in monomolecular reactions, which follow the principle of

mass action\*.

Besides these two variables there is also involved the variable activity of the catalyst; its activity is decreasing during the latter stages of the fermentation, due to the increasing concentration of the reaction products. The final equilibrium point of the reaction, or the ultimate zero slope of the fermentation curve, cannot be considered as primarily due to decrease in substrate concentration. While in ideal monomolecular reactions the final zero slope is due to this factor, the final concentration of the substrate in most agricultural lactic acid fermentations is not so greatly diminished at the end point of the reaction. It follows that the final zero slope can hardly be attributed to a diminution of the substrate concentration. (Compare "Principal Product of Lactic Acid Fermentation" --- "Amount of Lactic Acid Formed").

Then, during the course of lactic acid fermentation, the variable factors are the concentrations of the enzyme and of the substrate, and the activating influence of the enzyme. The speed of the reaction or the slope of the fermentation curve depends upon all of these factors, but these variable factors change in relative value throughout the course of the

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\* Probably all reactions follow the law of mass action, but other conditions may impose modifying influences upon the course of the reaction.

reaction. These and other conditions of lactic acid fermentation render most unsatisfactory any attempt to express the progress of the fermentation as the course of a first order chemical reaction.

### 3. Method of studying the "fermentation curve".

Possibly the fermentation curve could be considered as a composite curve of the curve of growth and of the monomolecular reaction induced by the lactic acid bacteria zymase, but difficult obstacles are again encountered in such an attempt. Although the enzymatic action might be considered as the fundamental reaction, the relative value of the two components of the fermentation curve will be changing throughout the course of the process. Probably, the progress of the reaction of lactic acid fermentation can be approached to best advantage by dividing the curve of its course into periods upon changes in reaction velocity shown by change in slope of the curve, or better still, by dividing the curve into arcs at points of inflection.

## V. Division of the "Fermentation Curve".

### 1. Basis of division.

Changes of slope of the fermentation curve indicate changes in the speed of lactic acid production; they may be considered as turning points in the lactic acid fermentation.

Still more significant are the points of inflection. Points of inflection of a curve separate

are concave upwards from those concave downwards; the derivative of the slope, or the second derivative of the curve itself ( $\frac{d^2LA}{dt^2}$  or acceleration) changes in sign at these points. They show acceleration or retardation of the reaction speed. These changes suggest a significant turning point in the progress of the fermentation caused by a change in the relative value of the determining conditions enumerated above and discussed below.

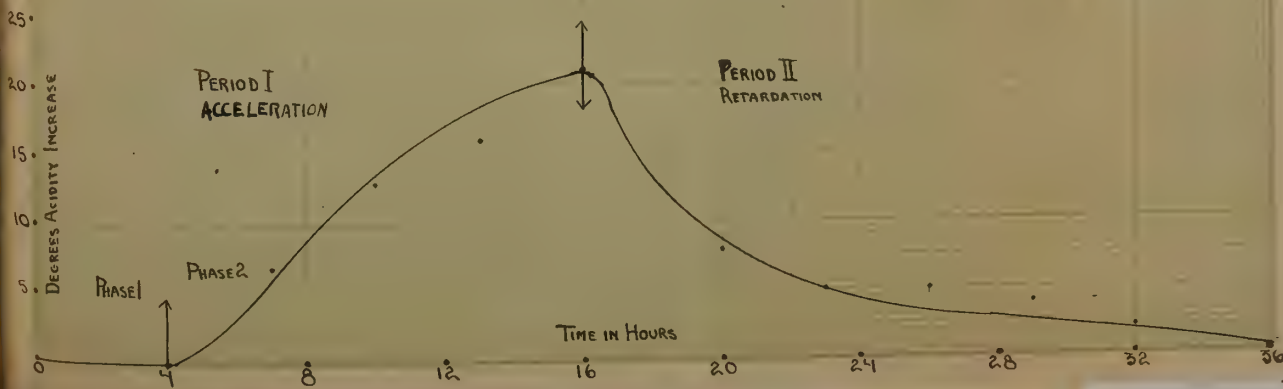
The changes in slope, and the inflection points may be seen in the above curves (Fig. 1), but they are still more evident in the rate curve given below. This "rate curve" has been derived from the same data as used in obtaining the fermentation curves of Fig. 1.

Figure 3.

"RATE CURVE"

showing Periods of Acceleration and of Retardation of the Progress of Lactic Acid Fermentations.

(FIGURE 3.)





## 2. Principles determining relative value of conditioning factors.

Before beginning a study of the different periods of the curve of lactic acid fermentation, a basis must be established by a statement of principles which determine the relative value of the influences imposed upon the curve by the above named conditioning factors.

### a. Increasing concentration of the catalyst.

The concentration of the enzyme may be considered a function of the number of lactic organisms present. The number of lactic acid bacteria is dependent upon the curve of growth. While multiplication need not conform strictly to the curve of organic growth, it will follow it to a certain extent during the early course of the fermentation. (Compare Figs. 1 and 2). That it does not follow the formula expression (of the curve of growth) introduces no error, as only the general form of the curve is of any moment in this discussion. The curve of growth is an exponential curve ( $N = Ce^{kt}$ );\*\* it is concave upwards throughout and consequently its slope is constantly increasing. It is not a factor throughout the reaction, but during the period in which it assumes importance the slope of the fermentation curve is increasing.

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\* See Fig. 4 from Rahn's work.

\*\* See Fig. 2.

The influence of this factor upon the slope of the fermentation curve is exerted through the relation of the concentration of the enzyme to the progress of the reaction; it will also be modified by the influence of the other factors.

This is evident by a comparison of the inflection point of the curve of growth (from Fig. 2) with the inflection point of the fermentation curve (from Fig. 1). This relation is clearly shown in Fig. 4.

#### Figure 4

(From data given by Rahn (1911), Table VII.)

#### Comparison Curve of Growth and Fermentation Curve

##### Interpretation of Curves:

###### Represents;

influence of growth and consequent concentration of enzyme upon the velocity of the fermentation.

###### Also shows;

similar response to unfavorable conditions exhibited by the fermentation reaction itself and by the growth of lactic bacteria.

the same influence apparently inhibits the fermentation reaction as inhibits growth.

###### Note;

inflection point on both curves is at practically the same concentration of the reaction product.

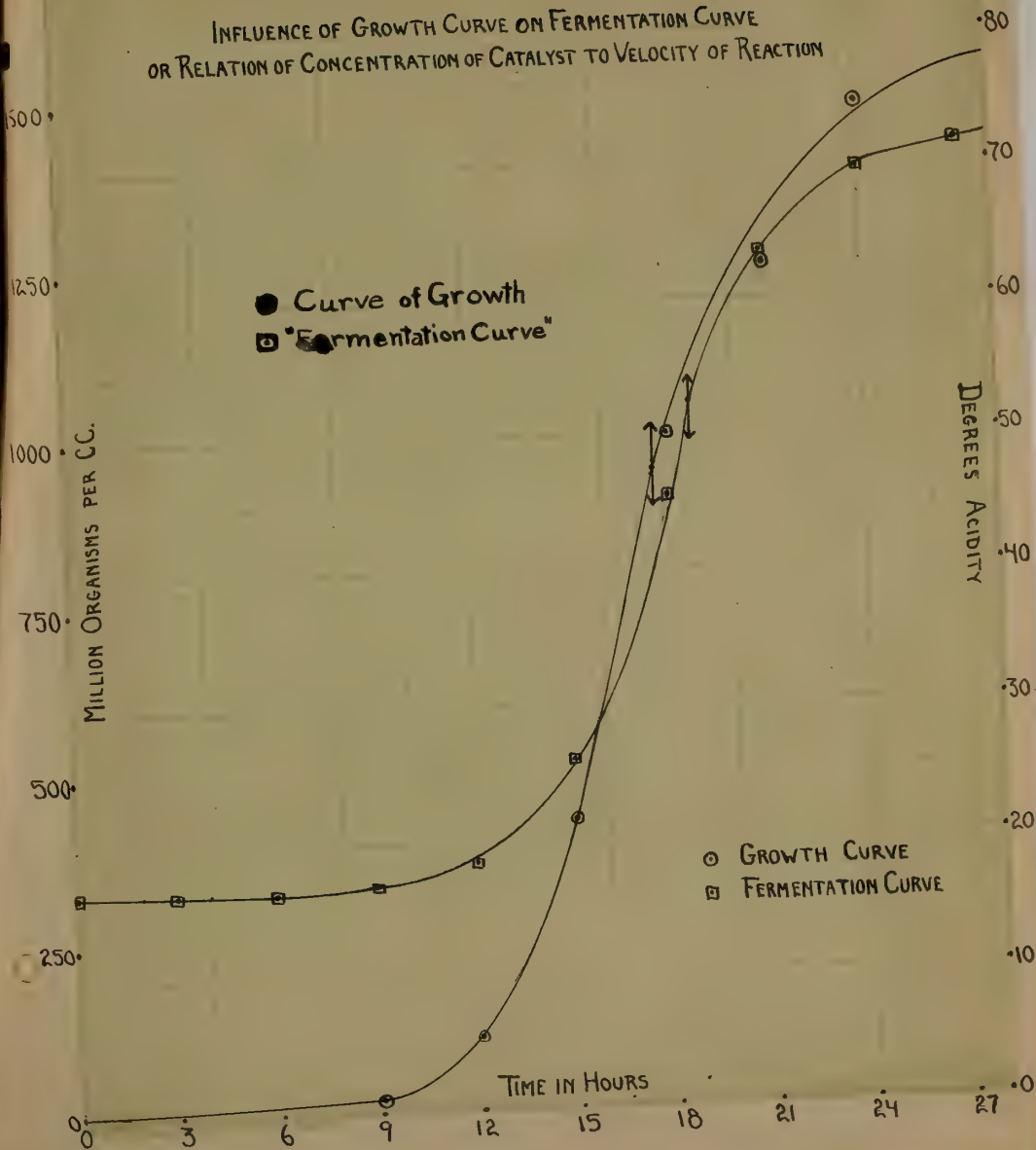
concave upward curve of growth has (during the period of its dominance) given a similar form to the fermentation curve. This relation is the cause of the acceleration exhibited in that arc of the fermentation curve.

When the fermentation curve becomes concave downward, retardation is exhibited by the change in sign of its second derivative.

Up to point B, the close agreement of the two curves strongly suggests that the velocity of the reaction (or slope of the fermentation curve) is proportional to the concentration of the catalyst.

FIGURE 4.

INFLUENCE OF GROWTH CURVE ON FERMENTATION CURVE  
OR RELATION OF CONCENTRATION OF CATALYST TO VELOCITY OF REACTION



b. Relation between concentrations of  
enzymes and of substrate.

The following principles have been established in the case of other similar catalytic reactions and may be assumed also to govern the progress of the reaction of lactic acid fermentation (Bayliss, Beatty, Duclaux, Brown, Arrhenius, Euler, Van Slyke and Cullen).

With constant and fairly high concentration of enzyme, monomolecular enzymatic reactions will obey the mass law of first order reactions; their velocity will decrease with the lowering of the concentration of the substrate. At any time the velocity will be a function of both, but with constant concentration of enzyme, it will vary only with the substrate concentration.

Even with constant concentration of enzyme the application of the mass law to enzymatic reactions is possible only if the enzyme concentration is not too low. If the substrate is in excess, the enzyme will be saturated with the substrate without producing an appreciable reduction of the concentration of substrate. This relation probably occurs during the early stages of lactic acid fermentation and exerts its influence upon at least the first part of the fermentation curve. (See Fig. 4, showing agreement in inflection points of multiplication and fermentation curve).



When substrate is in low concentration in the presence of an excess of enzyme, the velocity or slope will again be a function of one only, this time the concentration of the substrate. This relation manifests itself near the end of an ideal first order reaction, but it is doubtful if it is of much moment in most agricultural lactic acid fermentations.

That a similar effect on the course curve of the fermentation may be induced by other factors is shown in the following discussion of the influence of the reaction products upon the progress of the reaction.

#### c. Influence of reaction products.

The progress of a reaction is, in general,\* always retarded by the products of the reaction. (Compare Van Slyke and Zaccharias).

With enzymatic reactions another phenomenon tends to further decrease the velocity. It is a generally accepted assumption that enzymes enter into combination with the products of the reaction. In this way, the presence of a large concentration of the reaction products decreases the reaction velocity (or lowers the slope of the fermentation curve), not only as a condition of a reversible reaction, but also, and principally, by combination with, and inactivation of, the enzyme. This will produce an effect upon the form

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\* Exceptions may occur --- a notable example being cases of autocatalysis.

of the fermentation curve similar to that imposed by decreasing the concentration of the reacting substance in ideal unimolecular reaction curves. In lactic acid fermentation this is probably the most important factor influencing the slope of the latter part of the curve. (See Fig. 4, showing relation of multiplication to fermentation velocity).

The same conditions that lower the fermentation curve are lowering the speed of multiplication.

### 3. Choice of basis for division.

#### a. Grimm's division into phases on changes in slope.

Grimm has divided the progress of lactic acid fermentation into four more or less well defined phases based upon the rate of lactic acid production and bounded by the points of decided change in slope of the fermentation curve. Although Grimm's interpretation of the significance of all of these phases does not seem satisfactory, this division into phases corresponding to periods of the curve furnishes a convenient method of following the progress of a theoretical lactic acid fermentation. During these different phases, the progress of the fermentation is influenced to a different extent by the discussed factors. Naturally, the exact position of these phases varies in different fermentation curves and their boundaries are more or less indefinite even in the same fermentation. (See Fig. 1).

b. Sharper division on basis of inflection points.

The division of the fermentation into two stages at the inflection point of the curve is preferable in many ways and will be followed to a large extent in this discussion. Such a division seems to be a more fundamental one; the periods of acceleration and retardation of the reaction may be interpreted as expressions of the periods of dominance of those influences tending, respectively, to promote and to inhibit the progress of lactic acid fermentation.

The sharper division obtained by this method is evident in a comparison of the fermentation curves given in Fig. 1 and the rate curve shown in Fig. 3.

VI. Period of Acceleration (Concave Upward Arc).

1. Phase of gradual slope.

a. Zero or gradual slope.

In Grimm's curve (Fig. 1) it is seen that the plotting of lactic acid concentration during the first few hours after inoculation gives a fermentation curve parallel to the axis; the first part of the curve from Bohn's (1911) work also shows a very gradual slope, which for a longer distance from the origin is practically zero.\* This would indicate little, if any, increase in the lactic acid content of the system, provided that the

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\* Probably due to small inoculum.

methods of determining the lactic acid content of the medium presented a true account of the concentration of this reaction product. Similar form is seen in curves obtained by plotting the heat energy content (Rubner).

By these methods, then, of determining the course of the reaction, it would seem that the reaction made little or no progress during the first few hours after inoculation of the lactics into the medium.

b. Apparent discrepancy between growth of lactic acid bacteria and accumulation of reaction product.

This phenomenon was observed by Soxhlet as early as 1884. Furthermore, he found that there was an increase in the number of lactic acid bacteria during this period of apparent non-production of acid, which he termed the "incubation stage" of the fermentation. Many later investigations have corroborated Soxhlet's observation and "whatever the explanation, it is true that acid forming bacteria may increase until there are millions per c.c. and yet no change in acidity" can be detected. (Hastings, Evans and Hart).

The seeming paradox of delay of the lactic acid fermentation itself until after the beginning of multiplication has aroused considerable controversy. Rahn and other investigators disclaim the assumption of procedure of multiplication without fermentation.

The intimate relation of lactic acid fermentation to the metabolism of lactic acid bacteria and the fact that this process furnishes much of the energy required in all cell functions indicate that the fermentation would begin at the same time as multiplication and other life processes of the cell. Different explanations of this phenomenon follow.

c. Attempted explanations of the above discrepancy.

(1) "Incubation stage" of lactic acid fermentation.

Soxhlet (1884), Clauss, Plant, Knoesel (with yeasts in alcoholic fermentation), Rubner (by calorimetric method), Conn, Grimm (1912), and others have been led, by their observations, to believe that there is a certain period of "incubation" in the early history of fermentations, during which multiplication takes place before the reaction of lactic acid fermentation begins. Several facts vitiate this assumption of a certain period during which the cells increase in numbers and exert all life functions but the fundamental one of lactic acid fermentation.

The difficulty of measuring the small amount of lactic acid that would be produced by the few lactic acid bacteria\* present in the early period is certainly an important factor, as it casts doubt upon

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\* Compare text, under "Fermenting Capacity".



the authenticity of the data upon which the so-called "incubation stage" is founded. The influence of this factor is clearly seen in the results obtained by the supporters of this period themselves.

The length of the incubation period varies with the temperature, as would multiplication of the lactics, and consequent increase in concentration of catalyst.

Moreover, the length of time claimed by the early investigators for the incubation stage, as based upon lactic acid concentration of the medium, has kept decreasing in the later investigations as the methods of measuring the concentration of this product have improved. Although the lactic acid produced by a small number of lactics is very small, more delicate methods of measurement might reveal production of a certain amount during this apparently latent period.

## (2) Mechanism of lactic-acid-bacteria-*zymase*.

Other possible explanations, based upon phenomena associated with the method of action of the lactic-acid-bacteria-*zymase*, have been suggested. These, however, are not very satisfactory, as they represent nothing but speculations. It is possible that the cells retain the lactic acid within themselves and do not excrete this metabolic product until a certain time has elapsed. In view of the relatively large amount of lactic acid produced per cell in an hour, this does not

seem a plausible explanation. Possibly the combination between enzyme and substrate tends to delay the detection of lactic acid. The possibility of intermediate stages in the chemical reaction itself should not be ignored. Although these phenomena cannot in themselves account for the so-called "incubation stage", they may play a minor rôle by causing a slight delay in the liberation of lactic acid into the medium.

### (3) Incomplete data.

Rahn's (1911) explanation of the so-called incubation period furnishes the most satisfactory basis for the interpretation of the slope of the fermentation curve during this early phase. He believes that with acclimatized lactic acid bacteria, the process of lactic acid fermentation begins at once, but that the small amount of lactic acid produced by the few lactics is difficult to detect. The fact that with large inoculations this phase was shortened further strengthens his interpretation. This seems a much more plausible explanation than that lactic acid fermentation itself is delayed until after considerable multiplication of the cells has occurred.

### (4) Delay in multiplication.

In the case of unacclimatized or degenerate lactics, a delay in the fermentation process probably does occur but is then the result of a retarded state or condition extending to the other life process-

es as well. Many investigators of microbial life histories believe there is a period after inoculation into a new medium, during which the bacteria are more or less latent and that rapid multiplication begins only after a certain time has elapsed.

(a) Discussion of the so-called "lag period".

The so-called "lag period" of bacterial growth is intimately connected with this question. For this reason, the following interpretation of workers in this field is given below (Baseman (1895), Müller (1895), Rahn (1906), Rubner (1906, b), Lane-Claypon (1909), Penfold (1914), Ledingham and Penfold (1914), Slator (1916, 1917), Buchanan (1918), Barber ).

The earlier interpretation of the "lag" phase was that of a definite period during which growth was apparently in abeyance.

Later authorities interpret it more as a quantitative difference in rate or velocity of multiplication --- "a period which elapses between time of seeding and point at which the velocity of reproduction attains its maximal level."

With even this explanation, the fact cannot be ignored that the difficulty of measuring small increases in numbers of bacteria may enter and, to a certain extent, vitiate the data on which the existence of this phase depends. This is shown by the work of Chesney and of Barber, to be reported later.

(b) Significance in agricultural lactic acid fermentation.

In the progress of agricultural lactic acid fermentations a period of delayed or retarded multiplication is significant only when degenerate lactics are introduced as the fermenting agents.

"Lag" or "latent" periods are due either to extracellular or intracellular causes (Chesney). Extracellular causes would include any unfavorable environmental condition, such as hydrogen ion concentration, food, and temperature. In the early periods of

most important agricultural lactic acid fermentations the lactics usually find themselves in a favorable environment. Hence, the cause of any "lag" period must be found in the condition of the lactics themselves. Much evidence is presented that degenerate bacteria do exhibit a distinctly latent period when inoculated into a new medium. By direct microscopic observations, Barber found that no "lag" occurs with young acclimatized cultures of *B. coli*. Chesney has shown, that in the case of the pneumococcus, the "lag" which occurs with inocula of organisms weakened by previous exposure to metabolic products, is not manifested by inocula of vigorous cells transferred during the period of active growth.

Here the delay in multiplication, due to weakened activity of the lactics, together with the difficulty of detecting small amounts of lactic acid, would explain extended "incubation periods" in fermentations induced by degenerate lactics. The curves of such fermentations would show a very gradual or even a zero slope for a longer distance from the origin than would those in which vigorous cells were used as inocula.

#### d. Dismissal of this phase of the fermentation.

Since, in the case of active lactics, the existence of a so-called "incubation stage" seems to be based upon experimental error or upon observations obscured by limitations of technique, it is unwise to attempt an interpretation of the determining factors of this part of the fermentation curve. If accurate data were obtainable, it is probable that this phase of the curves of usual lactic acid fermentations would manifest the influence of the same conditioning factors as those determining the next phase.



## 2. Phase of rapidly rising slope.

This phase of the fermentation begins at point A on Grinn's curve; the position of this point is not so evident on the other curve. It is very probable that the division of the period of acceleration is based on experimental error; furthermore, it will be shown below that the factors determining the slope of the fermentation curve and the progress of the fermentation possess practically the same relative value during both of these proposed phases.

### a. Conditioning factors.

#### (1) Concentrations of substrate and of enzyme.

During this phase of the fermentation curve, the concentration of the enzyme is relatively low compared to that of the substrate. In the above discussion, it was stated that, under these conditions, Duclaux, Brown, and others found the velocity of the reaction or the slope of the curve to be dependent merely upon the concentration of the enzyme. This is explained (Brown, Bayliss) as being due to a combination between enzyme and substrate, and the consequent inability of a small amount of enzyme to affect more than a limited number of molecules of the substrate in a given time. This fact places the concentration of the enzyme as the dominant influence upon the slope of the curve during at least the greater part of this phase.



The concentration of the enzyme is dependent upon the growth of the lactic acid bacteria and consequently the concentration of the catalyst will be increasing more or less in general conformity with the law of organic growth.

The general form of the fermentation curves is in agreement with these deductions. It is concave upwards as would be the case with a curve largely dependent upon the exponential curve of growth. It is probable that the first phase is also largely an expression of the curve of growth. If accurate data were obtainable, it is probable that the point A would not exist on Grimm's curve (Fig. 1) and that this part of the curve would also be slightly concave upwards, with a gradual and then rapidly increasing slope as the exponential curve passed into Grimm's second phase.

## (2) Activity of the catalyst.

During this phase the total catalytic effect is greatest, as is evident from the high velocity of the reaction induced. As is seen in the curves, acceleration of lactic acid production occurs throughout, due to increasing concentration of the lactic acid bacteria zymase. The concave upward arc of the curve shows that even though a slight or local retarding influence is perhaps being exerted upon the activity of the enzyme by the presence of the reaction

products, the retardation is more than overcome by the accelerating influence of the increasing concentration of the catalyst.

b. Combination of first two phases into one period.

This phase of the fermentation Grinn terms "phase of increasing life activity of the lactic acid bacteria". Rahm, however, has shown that the activity or fermenting capacity of lactics is greater the younger the cell. Moreover, it would seem that the presence of even small amounts of the reaction product would retard, to a certain extent, the activity of the lactic acid bacteria and their zymase. According to this interpretation, Grinn's term "phase of increasing life activity" would include also his so-called "incubation stage".

The first two phases could be combined to form one period of the fermentation, based upon a fundamental property both in the fermentation curve and in the conditions existing in the system during this period. It would include the period of growth of the lactic acid bacteria and represent that part of the fermentation during which the reaction is accelerated due to the relative dominance of the conditions tending to promote the biochemical catalytic production of lactic acid. It could be termed "period of acceleration of lactic acid production" or "period of growth of lactic acid bacteria" (although the validity of the latter term may be questioned).

c. Practical significance of this period.

The practical application of an understanding of the existence of such a period during the course of lactic acid fermentation is seen in the use of lactic starters. Grimm found that the most desirable lactic acid fermentation of milk was produced by the use of inocula taken from cultures during this period of the life history of lactic acid bacteria.

d. End of dominance of accelerating influences.

During this period the fermentation curve steadily rises until the point of inflection B is reached. Although the curve is still rising, its slope or the speed of the reaction is less from that point than during the second phase. The point of inflection on the curves of different lactic acid fermentations will vary both as to time and acidity, according to the strain of lactic micro-organism and environmental conditions, but in all curves it is largely due to the decreasing influence of the concave upwards exponential curve of growth. In Rahn's (1910, 1911) work it occurred when acidity of  $50^{\circ}$  -  $60^{\circ}$  was reached; in Grimm's curve, when about  $70^{\circ}$  acidity is reached after about 16 hours' time from inoculation.

VII. Period of Retardation of Enzyme Action.

1. Phase of gradual inhibition.

a. Conditioning factors.

The point of inflection marks the elimina-

tion of the factor of increasing concentration of the enzyme; the reaction is now proceeding under the influence of a fairly constant concentration of the catalyst. The two determining factors of the slope of the curve are now the concentration of the sugar and the activity of the enzyme. The curve is now concave downward; the same form would be manifested near the end of the logarithmic curve of an ideal first order reaction, by reason of decreased substrate concentration. However, it is certain that the determining conditions of the velocity of the reaction are not of the same relative value in the case of agricultural lactic acid fermentations.

b. Concentration of substrate not the absolute factor.

The sugar concentration in usual lactic acid fermentation of milk is diminished only 20% during the entire reaction. During this phase, the diminution is about 6% of the initial concentration; this would decrease the total sugar concentration only 0.3%. If the substrate were the determining factor of the reaction velocity, the slope of the curve would not be greatly affected.

$$\frac{dx}{dt} = k(a - x) = k(5\% - x)$$

$$\frac{dx}{dt_1} = k(4.3\%) \quad \frac{dx}{dt_2} = k(4.0\%)$$

The slope of the fermentation curve, or reaction speed, at the beginning of this phase would be 43k; at the end, 40k, provided that the catalytic

action of the enzymes were unimpaired.

However, the gradual retardation of the velocity or decrease of slope is rather to be explained by the inhibition of the catalytic effect of the enzyme. The concentration of the reaction products is beginning to manifest its influence by inactivation of the enzyme, as explained in the above principles of enzyme action. The harmful effect of the products have, in earlier phases and perhaps in the first part of this phase, been lessened by the removal of the reaction product from the sphere of action of the enzyme by means of buffer effect of certain substances in the system. (Compare Van Slyke and Zaccharias).

### c. Characteristics of this phase.

This phase is characterized by its constant decrease of fermenting power. Active multiplication has largely ~~has largely~~ ceased; the reaction acceleration has been entirely lost and is supplanted by a gradual retardation; the slope of the curve decreases gradually until the point C is reached, which marks the beginning of the fourth phase.

## 2. Last phase of the fermentation.

### a. Actual inhibition of the reaction.

The slope of the fermentation curve has become practically zero and the curve becomes asymptote to the line 1a (Fig. 1), which represents the amount of lactic acid possible to be formed by this strain of lactic acid bacteria in infinite time under infinite-



ly optimum conditions. The insignificant velocity of the reaction is largely due to the inactivation of the enzyme by the products of the reaction; the biochemical catalyst has been "poisoned" by harmful substances in the system, analogous to Bredig's "poisoning" of inorganic catalysts. The factors in the inhibition of activity of the biochemical catalyst will be discussed in the following article.

Whether actual cessation of multiplication of the cells occurs before the action of the enzyme is absolutely inhibited is difficult to state. Grimm reports that the acid production ceases at the beginning of the fourth phase, but that slight multiplication still occurs. Rahn, however, found that, in his experiments at least, acid production continued after all multiplication had ceased. Here again, the question is obscured due to the difficulty of measuring small amounts of lactic acid that would be produced by the lactic acid bacteria in this stage (and still more so, the slight increase in number of cells). That the lactic acid fermentation process could proceed even after the death of the cells has been shown before. (See "Enzymes"). Here, however, the concentration of the reaction product is more in evidence. It is probable that this phenomenon is different under different conditions.

With proteolytic enzymes involved, instead of those acting on sugars, it is certain that enzymatic activity will continue long after multiplication has been inhibited. (In fact, in the case of the common lactic, it is only after autolysis that a significant attack on proteins is manifested). With enzymes such as the lactic acid bacteria zymase, the products of growth are probably as inhibitory to enzymatic activity as they are to the processes involved in multiplication. The greater resistance of proteolytic enzymes compared to that of carbohydrase enzymes is a quite general rule. (Avery and Cullen furnish a recent example in the case of the enzymes of pneumococci).

#### b. Practical significance.

During this phase of lactic acid fermentation the microbial activity is at its minimum. Transfers of the lactics from a system in this stage will not exhibit maximum physiological efficiency. Obviously, the use of starters from cultures of this age will not be advantageous, as shown by Rahrs (1911) and Grimm's experiments.

### VIII. End Point of the Lactic Acid Fermentation Process.

#### 1. Determination of end point.

The final end point of the fermentation is reached very gradually; probably, even after no further increase in acid production can be detected, the reaction is still proceeding at an inappreciable rate.

The actual end point is due to the inactivation of the catalyst; it is established at the point at which the retarding influences completely overcome those promoting production of lactic acid. It may be conveniently represented by an equation similar to that expressing Ohm's law, which Getman uses to explain ca-

alysis itself.

$$\text{Velocity of lactic acid production} = \frac{\text{driving force}}{\text{Resistance}}$$

When the retarding influences completely inactivate the "driving force" or the catalytic action of the lactic acid bacteria zymase, the velocity of the reaction becomes insignificant, which is the end point of the process of lactic acid fermentation.

## 2. Factors involved.

It is evident that lactic acid fermentation ceases when conditions in the system are such as to inhibit both the life processes of the lactics and the action of any enzyme that might be liberated by autolysis of the cells. The determining factor is probably different in different systems, but in all lactic acid fermentations some one, or a combination of the following factors must play the principal rôle; lack of available food in the medium or an accumulation of an inhibitory concentration of fermentation products.

### a. Utilizable food.

The principal food substances required by lactic acid bacteria are a fermentable sugar and utilizable nitrogenous material. In the more important agricultural lactic acid fermentations it is seldom that the sugar is completely exhausted (compare "Second Period of the Fermentation").

However, in many cases, as suggested by

Marshall,\* Rahn,\* and Hastings, Evans and Hart,\* the fermentation stops, due to the using up of the available nitrogenous food in the medium. For example, many lactic acid bacteria cease to multiply in milk before the accumulation of fermentation products has reached a prohibitive concentration. In these cases the end of the life history of the lactics is due to a failure in the supply of utilizable nitrogenous food, as is evidenced by a renewal of multiplication merely upon the addition of nitrogenous food in a form available to the organisms. (It is also possible that the supply of accessory food substances is exhausted).

#### b. Hydrogen ion concentration.

More often, however, the end point of the fermentation is brought about by an accumulation of fermentation products.

Whether the final cessation of lactic acid fermentation is in these cases due to the inhibition of the lactacidase (liberated and free in the medium or within the cell) or to inhibition of life processes of the lactics is impossible to determine, for reasons given before.

Chief among these fermentation products is lactic acid, although "other" fermentation products at times play a significant rôle. The principal inhibitory influence of the presence of this substance

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\* See "Microbial Association".

will be exerted by the hydrogen ion concentration produced by its dissociation in the medium. Examples of the influence of this factor, (which have been given in the discussion of "Influence of Hydrogen Ion Concentration on Lactic Acid Bacteria" ("Influence of Environment") ), show that in many cases the removal of this fermentation product by neutralization of the acid permits the fermentation process to continue. Hence, the end point of such agricultural lactic acid fermentations is determined largely by the high hydrogen ion concentration produced by the fermentation and the final  $pH$  value of the medium will be that of the fermentation limit of the lactic agent.

- c. Other products of the fermentation ---  
lactate ion, molecular lactic acid,  
and "other products" of unknown  
origin and nature.

In spite of the importance of the hydrogen ion concentration as a limiting factor, it is probable that other products of lactic acid fermentation at times prohibit the action of the lactic acid bacteria before the limiting hydrogen ion concentration is reached.

Brown (1914) attempted to explain the lower final titratable acidity produced in the lactic acid fermentation of complex sugars as "the result of some other decomposition products which accompany the acids, and, in connection with them, are able to inhibit growth". (Other statements in his report are not strictly in accord with the body of this article).

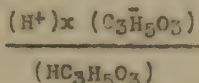


Rahn (1911) found that lactic organisms, which at first responded to neutralization by renewal of growth, failed to respond and decreased in numbers after repeated neutralizations. He believes this to be due to the concentration of sodium lactate or to "other products" of the fermentation.

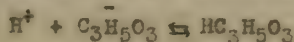
Clark (1915) interprets the following experiments upon the inhibitory influence of other fermentation products than the hydrogen ion concentration of the medium. Determinations were made of the final hydrogen ion concentration at which action of lactic acid bacteria of the first group ceased in media possessing different powers of neutralizing the acid and thus lowering the hydrogen ion concentration. He found that "in the more highly buffered media, that is, in the media with the greatest neutralizing power, growth stops at lower final hydrogen ion concentration and that this relationship is more or less orderly. .... The first conclusion to which this points is that, although the hydrogen ion concentration is the chief limiting factor, other toxic bodies, perhaps the undissociated acids themselves, .... accumulate during the fermentation and superimpose their own relatively small effect. .... Since the toxicity of these bodies would be proportional to their concentration, we should expect to find their effect greater in media which permit the more extended fermentation, namely in the highly buffered media."

The experiments of Von Dan also support the influence of other fermentation products than the specific effect of the dissociated hydrogen ion. From the results of experiments with *Strep. lacticus* in whey culture, he believes that in at least some media, (especially highly buffered media), the undissociated molecules of the lactic acid are a very important factor in the inhibition of growth by an accumulation of fermentation products.

He investigated the specific effect of the hydrogen ion and of the molecular lactic acid by an application of the ionization constant.



By adding soluble lactates to the whey medium, he displaced the equilibrium in the equation,



and suppressed the hydrogen ion concentration with a consequent increase in molecular lactic acid.

In these tests, growth stopped when molecular lactic acid reached 0.01 M, although the hydrogen ion concentration was considerably below the usual pH limit of this organism and could not, therefore, account in itself for the end of the fermentation.

In other experiments he added hydrochloric acid to the whey before inoculation. Here, growth ceased long before the concentration of molecular lactic acid could account for the inhibition; in these tests it was the specific effect of the hydrogen ion.

Evans and others have shown that the final pH reached by *Strep. lacticus* in yeast peptone sugar broth varies with the sugar substrate.

The influence exerted by substances similar to Northrup's "other products" should not be ignored. It is even more probable that lactic acid bacteria produce other unrecognized products of toxic influence to themselves than that they produce substances harmful to other micro-organisms.

The harmful effect of metabolic products of unknown nature, (some of them thermolabile), (Dykman (1904), Kruse and Pansini), is thought to be a factor in the inhibition of activity of probably all micro-organisms (Rahn (1906, 1917), Kruse (1910)). The elaboration of such products has been demonstrated in case of the colon bacillus and the pneumococcus. Cohen and Clark (1919) concede the possible significance of such substances in the inhibition of growth of acid formers.

In the above cases, practically nothing is known of the nature of these substances and indeed little more of their absolute effect. As to possible rôle of similar substances in the inhibition of activity of the common lactics, the work of Northrup is merely suggestive and by no means confirmatory. Therefore, any definite conjectures as to their rôle in the

end point of lactic acid fermentation is unwarranted. It is necessary, however, to bear in mind that the influence of the presence of these substances in the system may at times obscure the actual rôle of other factors, such as hydrogen ion concentration.

d. Relative importance of the hydrogen ion concentration factor.

All of this does not minimize in the least the importance of hydrogen ion concentration as one of the factors in the cessation of lactic acid fermentation. All lactic acid bacteria probably have limiting ranges of hydrogen ion concentration above which growth in any medium is prohibited. However, these upper limits can be considered as physiological constants only under like and definitely determined conditions. In spite of the fact that the hydrogen ion concentration is usually the most important factor in the determination of the end point of agricultural lactic acid fermentations, it is necessary to accept Clark's warning "that we must proceed with caution if we are to deal rigidly with the specific effects of the hydrogen ion".

e. "Prohibitory concentration product."

In view of the many factors shown to play a rôle in the cessation of lactic microbial activity,

is it not reasonable to assume that growth ceases when the sum total or product of all unfavorable conditions in the medium reaches a prohibitory concentration product? Some of the factors of this product are more important than others and may enter into the product as squares, cubes, or other powers; their relative importance would differ in different lactic acid fermentations.

In case food was not exhausted, the products of the fermentation and of the other life processes of the lactic organisms would be the chief factors in determining the end point of the fermentation. Among these, the hydrogen ion concentration would assume the greatest importance, (this factor probably possessing a high exponent), but in many cases it would not entirely suppress other factors of our "prohibitory product".

Example of cumulative effect of the "prohibitory concentration product" in cases where available food is not exhausted:

$$(\text{Sugar})^0 \times (\text{N})^0 \times (\text{H}^+)^{x^{\text{n}}} \times (\text{C}_3\text{H}_6\text{O}_3)^x \times (\text{"Other Products"})^y$$

$$= \text{K}$$

Raise exponent of the  $(\text{H}^+)$  factor and other products of K will be suppressed (probably the usual case in lactic acid fermentation).

Lower exponent of the  $(\text{H}^+)$  factor (as in Clark's and Van Dan's experiments) and other factors will have to increase to keep K a constant.



From this point of view, the lower final hydrogen ion concentration in highly buffered media could be explained by assuming that the higher concentration of other harmful factors, (e.g., undissociated lactic acid, "other products" of Northrup and probably still other unknown metabolic products of lactic acid bacteria), due to prolonged growth in these media, would require a lower hydrogen ion concentration factor to make the value of the product of all the factors reach what we have called the "prohibitory concentration product".

The inhibiting influences would be decreasing the speed of lactic acid production throughout the course of the fermentation; when their product reached the value K (which would be a constant only under strictly defined conditions of the experiment) the denominator of the proposed equation,

$$\text{Speed} = \frac{\text{driving force}}{\text{resistance}}$$

would be raised to a value reducing the rate of lactic acid production to insignificance. This, then, would mark the end point of the lactic acid fermentation reaction.

#### IX. Cases in Which Higher End Point is Attained.

In a study of the end point of lactic acid fermentation and of the factors determining the finish of the life history of lactic acid bacteria, two very interesting questions arise. In investigations of lactic acid fermentation it has frequently been observed that a higher final concentration of lactic acid is pro-



duced in media possessing seemingly minimum food, and still more often in fermentations held under temperature conditions considerably below the optimum for growth of the particular lactic acid bacteria involved.

These relations at first appear contradictory to the principles established in our discussion of the influence of environmental conditions upon the life processes of lactic bacteria. The following paragraphs have to do with the explanation of these phenomena.

#### 1. Influence of food upon rate of lactic acid production.

Rahn (1910) calls attention to the fact that poorly nourished lactic cultures maintain their activity for a much longer time after reaching their highest number and, in some cases, produce a higher final concentration of fermentation products. "The abundance of food in milk, cheese and similar products made the bacteria less resistant to their own products than the scanty food of soil extracts. . . . A few experiments indicate that poorly nourished bacteria are able to produce a larger amount of fermentation products than well nourished bacteria, although they need longer time to accomplish it." This phenomenon is in accord with similar observations in alcoholic fermentation.

Along quite the same line, Fred, Peterson and Davenport found that higher pH values are reached in the fermentation of carbohydrates that are attacked with difficulty. They ascribe this to the slow rate of the fermentation. Similar results are reported by other observers.

These phenomena are not contradictory to previous statements upon the influence of optimum conditions upon the life processes of lactic acid bacteria.

Such influences, if their effect is limited to the rate or speed\* of lactic acid production, should have no tendency to lower the final end point of the fermentation.

2. Influence of temperature upon the rate of lactic acid production and upon the final amount produced.

The second question may also be explained by plausible deductions from well established physical chemical principles. Under lower temperature conditions, as under "poorer" nourishment conditions, the rate of lactic acid production, (and also Rahn's "fermenting capacity"), is lowered. On the other hand, although the speed of lactic acid production is lowered, a higher final end point is attained. (See Fig. 5).

This relation --- higher final production of lactic acid in cases in which the velocity of the reaction is lower --- has frequently been observed. (Rahn, Schierbeck, White and Avery, Pennington, Jensen (1904) ). Higher accumulation of proteolytic products has also been found to occur in cultures held at temperatures below the optimum (Barthel,\*\* Gorini,\*\* and others).

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\* Compare curve of "Influence of Temperature" and its interpretation (Fig. 5).

\*\* See "Influence of Temperature".

Similar phenomena have been observed in alcoholic fermentation and are now applied in the industries.

Rahn (1917) explains this phenomenon "by the recognized experience that all products of fermentation tend to check its progress, and that any chemical product or substance acts the more vigorously upon any life process the higher the temperature." The same hydrogen ion concentration or the same concentration of metabolic products "that will still allow a slow fermentation at 10° may check the fermentation entirely at 20°."

It may likewise be assumed that our so-called "prohibitory concentration product" has a temperature coefficient which determines its inhibitory effect, not only upon the life processes, but also upon such enzymes as may be liberated upon death of the cells.

Compton's studies on the relation of hydrogen ion concentration of the medium to the optimum temperature of enzymes are suggestive of a possible future explanation of at least a part of this phenomenon. He (1915) has shown that an "increase in the hydrogen ion concentration in which an enzyme acts, beyond its optimum acidity, leads to a fall of the optimum temperature", and this (1921) independent of the concentration of the enzyme. It seems that this rela-

tion might also involve the maximum temperature, as well as the optimum, to enzyme action, thus making temperature a factor of moment in determining the final end point of fermentation reactions.

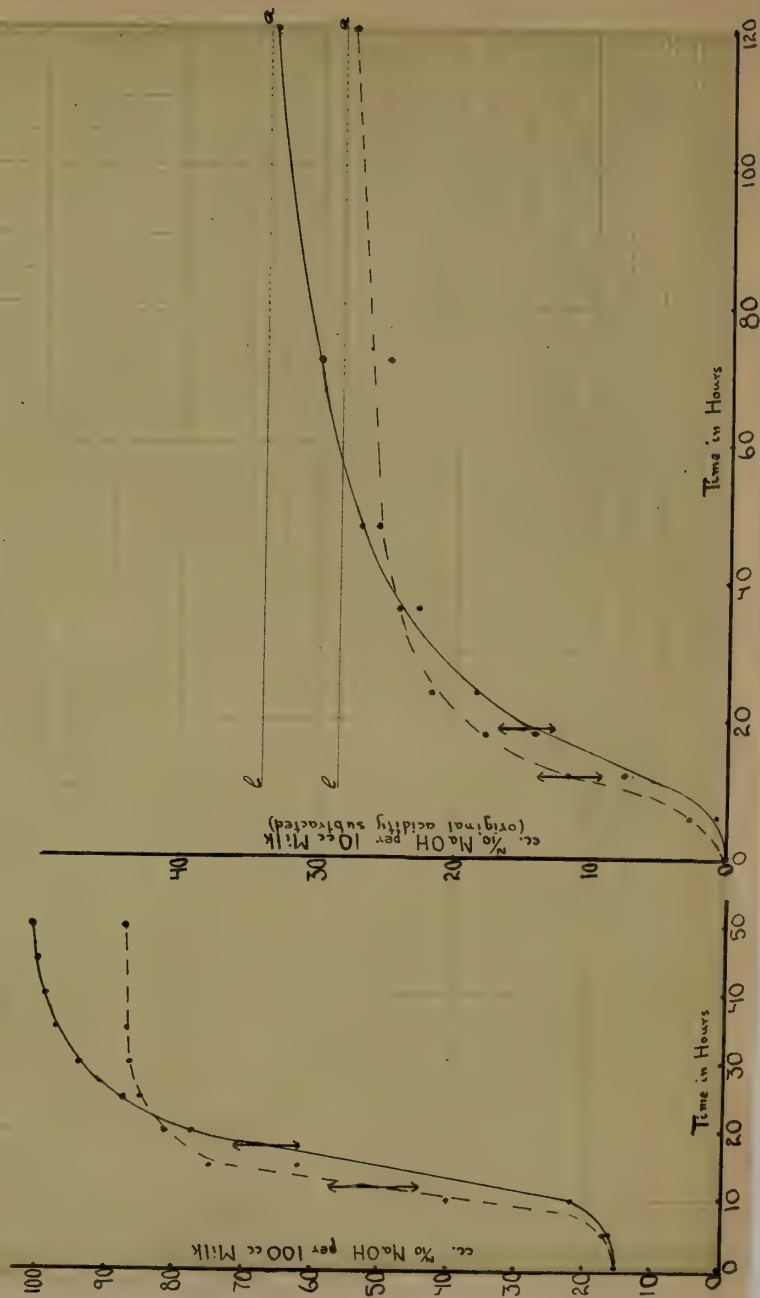
From a physical chemical standpoint, an application of the formula of Le Chatelier may be offered as a partial explanation of the higher final concentration of lactic acid reached in systems held at a low temperature. However, the fact that the equilibrium point of the reaction of lactic acid fermentation is a false equilibrium, probably decreases the importance of this factor.

Quite similar relations are observed in many inorganic catalytic reactions. The difference in the effect of temperature upon reaction velocity and final end point of chemical reactions is applied in the catalytic production of ammonia. At high temperatures, speed of reaction is greater, but at lower temperatures the final end point of this exothermic reaction more than counterbalances the advantages of greater velocity at higher temperatures. (Compare Henderson (1919) ).

The relation of velocity of lactic acid production to final amount produced is clearly shown in the curves presented in Fig. 5. Further discussion of this question is supplanted by an interpretation of those curves.

Figure 5.

# INFLUENCE OF TEMPERATURE UPON ENDPOINT OF LACTIC ACID FERMENTATION





### Figure 5.

Curves showing Relation of Temperature to Speed of Lactic Acid Production and to Final Concentration of the Product.

- I. From data furnished by Schierbeck.
- II. From data furnished by White and Avery (Tables I and II).

General characteristics of the curves:

Curves of fermentations at lower temperatures.

Gradual slope.

Slower rate, but higher final end point (line "la").

Curves of fermentations at higher temperatures.

Steep slope.

Greater velocity, but lower final end point (line "lb").

Significant features:

Slope of concave upward arcs;

In curves of the fermentation at higher temperatures, the slope is more steep. This indicates a more rapid increase in concentration of the catalyst, due to stimulation of growth.

Slope of concave downward arcs;

These arcs resemble logarithmic curves.

The slope of these arcs of the low temperature fermentation curves is less gradual than that of the arcs of higher temperature fermentation curves. This indicates that the rate of lactic acid production is greater throughout the later stages of the fermentations held at low temperatures than is the case in the higher temperature fermentations. This is due to a smaller value of

the inactivating influences, or to a delay in their action.

Concave downward arcs of the low temperature curves extend for a longer distance from both axes before becoming asymptote to the line "1a". This is due to the same causes as in the case of the differences in the slopes of these arcs. The line "1a" on the lower fermentation curve is further removed from the horizontal axis because of the lower value of the "prohibitory concentration product" at lower temperatures.

## X. Reversal of Reaction.

### 1. Reports of combustion of lactic acid itself.

The preceding discussion has been more or less limited to lactic acid fermentations in which the lactic acid bacteria were unable to attack the lactic acid produced. Frequent references are made in the literature of the combustion of lactic acid by the lactic acid bacteria which produce it. (Other following explanations would now possibly account for many of the reports of apparent direct combustion of lactic acid by the lactics.)

In his discussion of the theoretical progress of such lactic acid fermentations, Duclaux (1901) presents curves of the results of Kayser's experiments. The curves of the action of these lactic acid bacteria show that their life history and the progress of the induced lactic acid fermentations are different from those of the "true" lactic acid bacteria. In these cases, the curve of lactic acid production, after reaching a certain level, passes to a maximum and then, usually after some oscillation, begins to decrease.

The theoretical progress of such lactic acid fermentations is comparable to Rahn's (1910) representation of the progress of an associated fermentation of milk by lactic acid bacteria and the acid consuming *Oidium lactis*.

## 2. Fermentation of salts of organic acid.

### a. Simultaneous acid and alkaline fermentations.

In this connection the work of Ayers and Rupp (1918) seems very illuminating and offers a plausible explanation of the phenomena occurring in fermentations brought about by certain acid gas lactic acid bacteria\* and also, to a certain extent, by many other lactic organisms.

Their work has to do with the simultaneous acid and alkaline fermentations of members of our first group of lactic acid bacteria. It has been known for a long time (Pakes and Jollyman (1901), Harden (1901), and earlier workers\*) that some lactic acid bacteria are able to ferment the salts of formic and other organic acids. All of these workers have shown that these fermentations may yield alkaline products, (bicarbonates and carbonates), which will tend to lower the hydrogen ion concentration. "Since an organism can ferment sugar and form organic acids and, at the same time, ferment the salts of the same acids and oxidize these to alkaline carbonates, it is plain that these simultaneous fermentations may occur in any medium containing a fermentable sugar". (their immediate results were obtained in a synthetic medium).

They, (Ayers and Rupp (1918) ), emphasize the fact that these fermentations are simultaneous, for

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\* See Pakes and Jollyman for earlier references.

even in cases where no organic acid salts are initially present in the medium, "as soon as organic acid salts are formed from the sugar they are immediately fermented, after which the two fermentations progress simultaneously." In the usual agricultural lactic acid fermentations "the reaction may be due to the acid fermentation of the sugar and an alkaline fermentation, not only of the organic acid produced from the sugar, but those in the medium before inoculation."

b. Significance in agricultural lactic acid fermentation.

In view of the significance of the reaction of the medium upon the physiology and life history of the lactic acid bacteria, it is evident that the above phenomena may, in many cases, be of significant moment in determining the progress and direction of agricultural lactic acid fermentations.

c. Significance in the interpretation of the presence of the colon-aerogenes group in various products.

In the discussion of the acid gas group of lactic acid bacteria, it was shown that the important members of this group have been divided into two quite well defined sub-groups, based upon the correlation of several tests. All of these tests are based upon fundamental and apparently deep seated differences in the metabolism of the members of the two sub-groups.



A report on the development of the interpretation of the "methyl red test" is given below. There, it will be seen that the differences exhibited by this test are based upon differences in the direction and rate of progress of reactions which are intimately connected with the chemical changes already shown to be brought about by different lactic acid bacteria of the acid gas type.

#### X. (Addendum).

##### "Methyl Red Test".

Although a full discussion would be out of place in this paper, it is interesting to note the phenomena occurring during the progress of certain acid gas lactic acid fermentations, which furnish the basis of the "methyl red test" for differentiation between the "low gas" ratio and the "high gas" ratio lactic acid bacteria.\* Clarke and Lubs devised this useful diagnostic test by "imposing conditions under which the metabolism of these (lactic acid bacteria) can be so controlled that the hydrogen ion concentration of cultures of one group can be made to diverge widely from those of the other group."

They, (1915), explain it as follows: "All organisms of colon-aerogenes group which give low gas ratio will, if furnished sufficient fermentation carbohydrate, continue to elaborate acid until a certain zone of hydrogen ion concentration is reached. There all activity ceases. The particular point in this zone is determined by the nature of the medium, but in any medium the particular point reached is remarkably constant."

On the other hand, "in media in which low ratio organisms reach their limiting hydrogen ion concentration, high ratio organisms are found to reach a much lower value, between  $pH$  6 and  $pH$  7. It must not be inferred that high ratio organisms cease fermenta-

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\* See "Groups of Lactic Acid Bacteria".

tion at such low hydrogen ion concentration because they can endure no higher.\* Rather does it appear that these organisms have fermented all the sugar, (the medium for this test contains but 0.5 % dextrose), with production of a quantity of acid insufficient to inhibit further growth. Increase the dextrose and, up to a certain limit, the hydrogen ion concentration is increased. From this it is evident that the low values of hydrogen ion concentration in dilute sugar cultures of high ratio organisms are not limiting values." \*

In the particular medium used, the final hydrogen ion concentration of these two sub-groups of lactic acid bacteria are pH 5 and pH 6-7. Therefore, the two may be readily differentiated by use of the indicator methyl red.

Ayers and Rupp (1918) explain the reversion of reaction, (upon which the recognition of these groups is based), by the alkaline fermentation of salts of the organic acids produced. The specific course which this process follows depends upon the organism and the constituents of the medium. In the case of these two sub-groups of lactic acid bacteria and in the medium used in the methyl red test its direction is as follows:

The acid gas lactic bacteria produce large amounts of formic acid\*\*which forms salts with the buffer substances of the medium. The formate salts are attacked with production of bicarbonates; these then react with the acid phosphate in the medium, liberating carbon dioxide and forming  $K_2HPO_4$ ; this salt reacts alkaline (by hydrolysis) and causes a reversion of the reaction. "The essential process, and the one on which the other adjustments of equilibrium depend, is the replacement of a relatively strong acid (formic acid) by the relatively weak carbonic acid." The difference in dissociation of these two acids naturally causes a lowering of the hydrogen ion concentration.

Colon and aerogenes cultures both produce a simultaneous fermentation of the dextrose and the organic acid salts, under proper conditions; the reason the hydrogen ion concentration reverts, in the case of the aerogenes cultures, is because a different relation

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\* Recall statements given under "Influence of Hydrogen Ion Concentration", showing that the aerogenes group can reach a pH limit of 4.5 in other media.

\*\* See "Acid Gas Group" of "Lactic Acid Bacteria"; also "Volatile Acids" in "Other Products".

exists between the speed of acid fermentation of the dextrose and the speed of alkaline fermentation of the organic acid salts in the life history of these organisms than in that of the colon group. "The difference between the colon and aerogenes cultures is one of rate, the final hydrogen ion concentration being the result of the rates of the acid and alkaline fermentations."

Some investigators, (Berman and Rettger (1914), Klegler (1916), Levine (1914) ), have believed this reversion to be due to neutralization of the acid by means of ammonia formed by decomposition of protein substances. The above work of Ayers and Rupp and that of Clarke and Lubs (1917) tend to disprove this assumption. The fact that in their work they obtained a reversal of reaction in media containing no substance from which ammonia could be formed tends to substantiate Ayers and Rupp's explanation given above.

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#### H. THE PRINCIPAL PRODUCT OF LACTIC ACID FERMENTATION.

- A. Lactic Acid as a Chemical Substance.
- B. Stereochemical Lactic Acid Fermentation.
- C. Amount of Lactic Acid Formed.

## A. LACTIC ACID AS A CHEMICAL SUBSTANCE.

### I. History of Lactic Acid.

1. Recognition of lactic acid as a distinct acid.
2. Nature and composition of lactic acid.
3. Distribution and occurrence of lactic acid.

### II. Systematic Position of Lactic Acid.

1. Lactic acid,  $\alpha$ -hydroxy propionic acid.
2. Relation of lactic acid to other propane derivatives.
3. Reactions of synthesis of lactic acid.

#### a. General methods.

#### b. Non-biological production of lactic acid from lactic acid fermentation substrates.

4. Reactions of decomposition of lactic acid.

### III. Properties of Lactic Acid.

### IV. Qualitative Determination of Lactic Acid.

1. Examination; physical, chemical tests.
2. Davis's method for separation of lactic acid.
3. Tests upon oxidation products.
4. Other reaction tests.
5. Microchemical detection of lactic acid.

V. Quantitative Determination of Lactic Acid.

1. Acidimetric methods --- in absence of other acids.
2. Methods used in analyses of fermentation mixtures.
  - a. General preliminary procedure.
  - b. Palm's method.
    - (1) Basis.
    - (2) Procedure.
    - (3) Value.
  - c. Partheil's distillation method.
    - (1) Basis.
    - (2) Procedure.
    - (3) Value.
  - d. Zinc lactate method.
    - (1) Basis.
    - (2) Procedure.
    - (3) Value.
  - e. Determination of lactic acid in the presence of succinic and other "fixed" acids.
  - f. Indirect determinations of lactic acid.
  - g. Factors to be considered in the quantitative determination of lactic acid.
3. Determination of "free" lactic acid.
  - a. Conditions of existence of lactic acid.
  - b. Methods of determining the amount of "free" lactic acid.
    - (1) Hydrogen electrode.
    - (2) Partition coefficient.
    - (3) Electrometric titration.

## VI. Stereochemistry of Lactic Acid.

### 1. History.

### 2. Distinction between active and racemic forms.

a. Requirements of method of procedure.

b. Properties of the lactates:

water of hydration.

$\text{H}_2\text{O}$  content.

optical character.

c. Manipulation.

### 3. Distinction between dextro lactic acid and levo lactic acid.

a. Properties of the acids.

b. Properties of the lactates.

c. Manipulation.

### 4. Resolution of racemic lactic acid into its two optically active components.

a. Pasteur's general methods of resolution.

b. Mechanical separation or resolution by crystallization in enantiomorphous forms.

c. Method of formation of derivatives with optically active substances.

d. Biological method of resolution.

### 5. Racemization or transformation of active lactic acids to the racemic form.



## THE PRINCIPAL PRODUCT OF LACTIC ACID FERMENTATION.

### A. Lactic Acid as a Chemical Substance.

#### I. History of Lactic Acid.

##### 1. Recognition of lactic acid as a distinct acid.

The establishment of lactic acid fermentation as a distinct and individual fermentation process was directly dependent upon the recognition of lactic acid as a distinct acid. The obvious dependence of the interpretation of lactic acid fermentation upon the chemical interpretation of its principal product is strikingly evident in the more or less parallel developments of the two interpretations. The history of lactic acid also shows, however, that many valuable contributions to chemical developments are likewise furnished by microbiological investigations in related fields. (These relations will be evident to the reader by a chronological comparison of the following paragraphs on the history of lactic acid; with the history of the interpretation of lactic acid fermentation given in the first chapter.)

In a report to the Stockholm Academy in 1780, Scheele described the properties of an acid which he had found in sour milk. This acid he termed acidus lactis or galacticum. (Kopp).

The identity of the lactic acid reported by Scheele was accepted for a number of years. However, it was soon attacked by a group of chemists who were laboring under the then prevailing impression that all organic acids were simply modifications of the simpler acetic acid (an impression which was a legacy from the last of the Phlogistic school) (Kopp).

Scheele had observed the similarity of his acidum lactis to acetic acid, and later to malic acid, but he claimed the sour milk acid to be a distinct substance by reason of the properties of its calcium salt.

Lavoisier (1792) denied the individuality of lactic acid and claimed it to be merely "incomplete" acetic acid. Bouillon-Lagrange<sup>#</sup> (1804), and Fourcroy and Vauquelin<sup>#</sup> (1806) reported their beliefs that lactic acid was merely "masked" acetic acid, the true properties of which were disguised by a portion of extractive matter united with it, and by the saline constituents of the whey.

Berzelius discovered an acid in muscle juices (1808) and in other animal liquids (1813), which he claimed to be the lactic acid of Scheele (ref. Kopp). For a time he was an active defender of the identity of lactic acid. However, a few years later, Berzelius (1822) inclined to the opinion that lactic acid was probably a combination of acetic acid and animal matter. (ref. Kopp and Henry). (Berzelius himself later claims that he never denied the difference between the two acids. (ref. Huetppe)). In 1829, Berzelius returned to the defence of lactic acid and announced (in opposition to Gmelin) that lactic acid was a distinct and individual acid.

Braconnot (1813), working at Nancy, found that a certain acid was formed in fermented rice, beet juice and similar substances. He believed this to be a distinct acid and called it "acide pancéique". Probably largely due to the, at that time, disputed identity of lactic acid itself, he does not recognize an agreement between his "acide pancéique" and Scheele's lactic acid. The agreement between the two acids was shown by Vogel in 1818.

The confused status of lactic acid as a definite substance is evident in the statements on the "so-called lactic acid" and "modified acetic acids",

<sup>#</sup>These chemists also attacked the identity of other organic acids. Bouillon-Lagrange, together with Vogel (1807), claimed that malic and gallic acids were also only "modified" acetic acid; Fourcroy and Vauquelin made the same claims against formic acid and pyrotartaric acids. (ref. Kopp).

which appear in chemical text books during the first three decades of that century. (e. g., Henry (1814, 1829); Murray (1819)).

The definite establishment of lactic acid as a distinct and individual acid awaited the following better controlled studies of its composition. Liebig and Mitscherlich (1832) and Pérouze and Gay Lussac (1833), by careful analyses of its salts, definitely proved the identity of lactic acid. (The latter authors also confirmed the identity of lactic acid and Bracconnet's "acide nancéique".)

## 2. Nature and composition of lactic acid.

Following the above establishment of the individuality of lactic acid, several controversies were involved in the establishment of its composition and nature.

A number of important investigations by Liebig, Engelhart, Heintz, Wisclenius, and others, (from 1847 to 1863), contributed to our knowledge of the composition and nature of lactic acid. Their studies, however, are concerned most directly with the stereochemistry of lactic acid and are reported later in this section of the present chapter.

Liebig had determined the composition of lactic acid to be  $C_3H_6O_3$ . Several years later Engelhart and Maddrell studied a number of salts of lactic acid and reported that it was a dibasic acid, giving acid salts. Gerhart also regarded lactic acid as a dibasic acid. Under the influence of this

belief, its formula was doubled. However, this conception of the composition of lactic acid was soon reversed by Strecker's synthesis and the older formula of Liebig was readopted.

A spirited dispute over the basicity and nature of lactic acid occurred between 1858 and 1860 (largely conducted between Wurtz and Kolbe).

Wurtz (1858) reported his belief that lactic acid was dibasic, due to its relation to α-propylene glycol.

Kolbe (1859) considered it to be a monobasic acid. By analogy with similar relations in the case of other hydroxy acids, he believed that lactic acid should be considered as hydroxy propionic acid. (Ulrich's report of the reduction of lactic acid to propionic acid offered further evidence in support of this contention of Kolbe.)

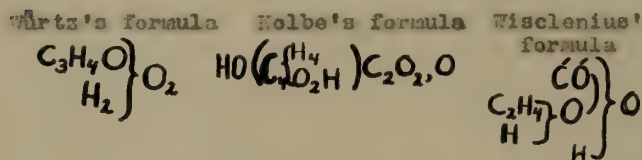
Wurtz in 1860 introduced a cert in distinction between atomicity and basicity, and called lactic acid a diatomic, monobasic acid. (This conception merely indicated that one of the two typical hydrogen atoms was more basic than the other; and even this was not expressed in Wurtz' typical formula. See below.)

Wisclenius (1860) reconciled the views of Kolbe and of Wurtz by suggesting that both alcohol and acid properties were possessed by and within the lactic acid molecule. These relations on the nature of lactic acid, however, were much more clearly expressed by Kekulé who showed that lactic acid is both an alcohol and an acid.

The formulae given below are suggestive of the above disputes over the basicity and nature



of lactic acid. The "type" formular notation persisted in the case of lactic acid, as for other compounds. The true significance of Kekulé's and of Couper's views was not applied until some time after their proposal, as is evident from the above controversy over the basicity of lactic acid (which Kolbe and Würtz began even after the advancement of Kekulé's (1858) and of Couper's (1858) views).



These controversies established the composition formula for lactic acid, its relation as hydroxy propionic acid, and its nature both as an acid and alcohol. Later the work of Erlenmeyer (1866) and Frankland (1867) led to the adoption of the modern method of notation and its application to the present structural formula of lactic acid.

### 3. Distribution and occurrence of lactic acid.

Lactic acid was reported present in sour milk by Scheele (1780); in muscle and other animal juices, by Berzelius (1807, 1813); in fermented rice and similar substances by Bracconnet (1813); in beet juices, etc., by Pérouze and Gay Lussac (1833); in gastric juice, by Bernard and Berreswil; in egg yolk, by Gobley; in beer, by Wackenroder (1846).

During the early history of the study of lactic acid, material for investigation was commonly



obtained by chemists from the fermentation of sugars (even before the definite establishment of lactic acid fermentation as a distinctive fermentation process).

Lactic acid has later been reported present in barley, corn and potatoes, (Windisch, 1887); it has been found to be a common constituent of the juices and extracts of many plants (references cited by Wakschmann). It is now considered as a normal constituent of wine (Balard, 1841), of molasses (Schöne and Tollens, 1900), and of opium (Smitt).

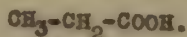
At the present time lactic acid is recognized as occurring in and entering into a very large number of important agricultural processes.

## II. Systematic Position of Lactic Acid.

### 1. Lactic acid, $\alpha$ -hydroxy propionic acid.

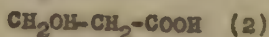
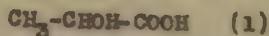
Lactic acid is an hydroxyl substitution product of propionic acid; its systematic name, is  $\alpha$ -hydroxy propionic acid, with the composition formula  $C_3H_6O_3$ .

With propionic acid there are two carbon groups in which the OH group could be substituted, as seen in the formula



(2) (1)

The OH group could be substituted either in carbon group (1) or (2); these substitutions would result in the formation of the following OH acids:



These acids are named systematically, according to the relative position of the COOH group to the carbon group in which the OH substitution took place,--- *a*-hydroxy propionic or *B*-hydroxy propionic acid. That these two acids are chemically different has been conclusively proven by the reactions by which each is synthesized, by the products they yield on oxidation, and by other reactions, such as the failure of hydracrylic acid to give any trace of lactide upon heating.

It is with the first of these hydroxy propionic acids that we are concerned, that is, with *a*-hydroxy propionic acid, or ethylidene lactic acid. Lactic acid fermentation always\* yields this acid and not the *B*-hydroxy propionic or, as commonly termed, the hydracrylic or ethylene lactic acid.\*\*

\*\* The use of the term "ethylene lactic acid" in referring to *B*-hydroxy propionic or hydracrylic acid is in disrepute. Systematic organic chemists justly complain that this substance is not a lactic acid at all.


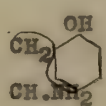
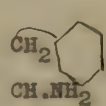
2. Relation of lactic acid to other biologically important propane derivatives.

Lactic acid is  $\alpha$ -hydroxy propionic acid. It is, therefore, closely related to other propane derivatives. Its structural relation to some of its chemical relatives is shown by the following formulae.

$\text{CH}_3$	$\text{CH}_3$	$\text{CH}_3$	$\text{CH}_3$
$\text{CH}_2$	$\text{CH}_2$	$\text{CO}$	$\text{CO}$
$\text{CH}_3$	$\text{CH}_2\text{OH}$	$\text{CH}_3$	$\text{CHO}$
Propane	Propyl alcohol	Acetone	Methyl glyoxal*
			or Pyruvic aldehyde

$\text{CH}_3$	$\text{CH}_2\text{OH}$	$\text{CH}_2\text{OH}$	$\text{CH}_2\text{OH}$
$\text{CHOH}$	$\text{CHOH}$	$\text{CHOH}$	$\text{CO}$
$\text{COOH}$	$\text{CH}_2\text{OH}$	$\text{CHO}$	$\text{CH}_2\text{OH}$
Lactic Acid	Glycerol	Glycerine aldehyde*	Dioxyacetone*

$\text{CH}_3$	$\text{CH}_2\text{OH}$	$\text{CH}_2\text{SH}$	$\text{CH}_3$
$\text{CH.NH}_2$	$\text{CH.NH}_2$	$\text{CH.NH}_2$	$\text{CH.SH}$
$\text{COOH}$	$\text{COOH}$	$\text{COOH}$	$\text{COOH}$
Alanine	Serine	Cysteine	$\alpha$ -Thiolactic acid

			Indol group
$\text{CH.NH}_2$	$\text{CH.NH}_2$	$\text{CH.NH}_2$	
$\text{COOH}$	$\text{COOH}$	$\text{COOH}$	
Phenyl alanine	Tyrosine	Tryptophane	

\* Proposed intermediate substances in lactic acid fermentation.

Many of these substances assume importance in physiological processes. Of these the most important are: lactic acid, acetone, glycerol, glyceric aldehyde, dioxycetone, alanine, serine, cysteine, tyrosine, and tryptophane.

### 3. Reactions of synthesis of lactic acid. \*

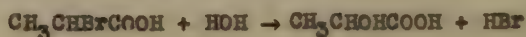
Lactic acid is a monohydroxy acid. All of these acids may be considered as OH substitution products of acids, or as derivatives of alcohols, in which one of the hydrogen atoms has been replaced by a COOH group. They contain both the CHOH alcohol group and the COOH acid group. The methods used in the syntheses of these acids are applications of this view of their structure. Following are important methods of synthesis <sup>of  $\alpha$ -hydroxy acids as applied to the synthesis</sup> of lactic acid. \*\*

\* Lactic acid is not prepared commercially by any of these reactions of synthesis, but by lactic acid fermentation of sugars. For details of its commercial preparation, see the following references:

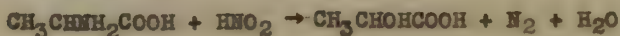
- Clafin, J., Soc. Chem. Ind., 1897.  
XVI, 516.  
Shafer, Chem. Zeit., 1907, VI, 177, 189.  
McLauchlan, Int. Congress Appl. Chem.,  
1909, Section IVA, I, 141.  
Molinari (1913), Martin (1918).

\*\* Note that some of these reactions, too, are induced by catalysts.

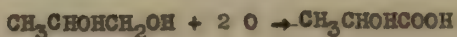
Action of  $H_2O$  on halogen substituted acids.



Action of  $HNO_2$  on  $\alpha$ -amino acids.

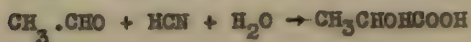


Limited oxidation of a polyatomic alcohol.

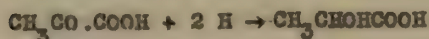


(Oxidation with platinum black)

Hydrolysis addition products of aldehydes and ketones with HCN.



Reduction of compounds which contain both a COOH and a CO group.



(Pyruvic  
acid)

More pertinent, however, are the purely chemical means by which it may be formed from these same substances from which it is derived in lactic acid fermentation. The formation of lactic acid by action of alkalis upon sugars has been reported by a number of investigators.

Nencki and Sieber report formation of lactic acid from dextrose with only 0.3 % KOH. Kiliani reported formation of lactic acid by action of alkalis occurs more readily in absence



of air or oxidizing substances. Nef also observed alkali splitting of dextrose, with formation of methyl glyoxal\* as an intermediate substance. Buchner and Meisenheimer observed splitting of dextrose by 5% KOH with formation of lactic acid, at room temperature, in diffuse daylight and even in the dark. Although the reaction is slow, after eleven months all of the dextrose was transformed to lactic acid.\*\* Meisenheimer later found that galactose also yielded lactic acid by action of alkalis, although less amounts were formed than in the case of glucose.

Strecker proved that lactic acid can be formed from the amino acid, alanine ( $\text{CH}_3\text{CHNH}_2\text{COOH}$ ), by simple deamidization and oxidation.

\* Compare "Intermediate Products" in "Chemical Changes Involved in Lactic Acid Fermentation", p.

\*\* It is a common laboratory observation that sugar media, especially dextrose broths, tend to become more acid if left standing for some time. Very possibly this is due to a similar reaction of slow velocity upon the sugar, resulting in formation of small amounts of lactic acid.

#### 4. Reactions of decomposition of lactic acid.

The most important reactions of decomposition of lactic acid are the following:

Heated at  $140^\circ$ , the anhydride is formed.\*  

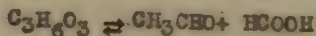
$$2 \text{C}_3\text{H}_5\text{O}_3 \rightleftharpoons \text{C}_6\text{H}_{10}\text{O}_5 + \text{H}_2\text{O}$$

Lactic acid, evaporated at ordinary temperatures, in dry air, forms lactic anhydride and lactide in proportions varying with time of deacid-

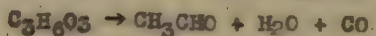
cation. (Davis).

Heated at temperatures above  $140^{\circ}$ , volatilization and splitting into  $H_2O$ ,  $CO_2$ ,  $CH_3CHO$  and lactide ( $C_6H_8O_4$ ).

Heated with dilute  $H_2SO_4$  at  $130^{\circ}$  \*\*

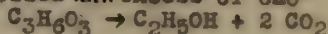


Heated with concentrated  $H_2SO_4$  \*\*



Heated with alkalis, lactic acid yields members of the acetic series and also higher unsaturated acids. (Hoppe-Seyler(1878), Raper(1905).

Distilled with excess of  $CaO$



Lactic acid is oxidized by  $H_2O_2$  to  $CH_3COOH$

Lactic acid is oxidized by molds to final oxidation products. \*\*\*

Digested with nitrophenylhydrazine at  $37^{\circ}$ , lactic acid is converted to methylglyoxal<sup>#</sup> (Dakin and Dudley).

\*\* Compare "Indirect Methods of Determination of Lactic Acid".

\*\*\* Compare "Energy Transformation".

# Compare "Trioses as intermediate substances" (under "Chemical Changes").

### III. Properties of Lactic Acid.

In a completely pure state, lactic acid is a crystalline solid possessing specific gravity 1.23 (25° C.)<sup>(Dyes)</sup> and melting point 18°. However, it is almost always obtained as a colorless or slightly yellow syrup.\*

It is a monobasic acid as it possesses but one COOH group. Its dissociation constant after Ostwald is 0.000138,<sup>(ref. van Dam)</sup> Michaelis, 0.000135; according to other investigators, this is too low a value.

Lactic acid is soluble or miscible in all proportions in water, alcohol, glycerol, and ether.<sup>(Davis)</sup> Its solubility in the last named solvent furnishes the basis of its extraction from many of its agricultural sources. When extracted from H<sub>2</sub>O by ether (in absence of interfering substances) its distribution coefficient

\* Commercial lactic acid is usually sold in this country in the form of a solution with an acidity representing 25% lactic acid. A 50% solution is more common in England and in Germany. The lactic acid of the British and U. S. Pharmacopoeia has specific gravity of about 1.21 and should contain 75% lactic acid. It also contains about 8% lactic anhydride. (Davis(1913, 1917), Molinari(1913), Martin (1918)).

Very pure lactic acid may be obtained by extracting the crude product with amyl alcohol (which the usual impurities, - sugar, gum, mineral substances - are insoluble) and distilling in vacuo. (Molinari)

(Van Slyke + Baker)

ient is 0.08 at usual temperatures. \*\* Lactic acid is slightly soluble in chloroform, and insoluble in carbon disulphide and petroleum spirit. (Davis)

The acid forms lactates with bases. The most important of these are the Ca, Zn, and Li salts, all of which are hydrated. The Zn salts of active and racemic lactic acid possess definitely different amounts of water of hydration, and consequently yield different amounts of ZnO upon ignition.

All the metallic lactates are more or less soluble in water, but usually dissolve only sparingly in the cold. All are insoluble in ether. Davis gives detailed properties of the calcium, ferrous, lead, zinc, and magnesium lactates:

\*\* See "Quantitative Determination of Lactic Acid" for influence of this low coefficient upon methods for quantitative separation of lactic acid.

#### IV. Qualitative Determination of Lactic Acid.

Qualitative determinations of lactic acid are made by characteristic reactions based on its chemical relationships; by isolation of the lactic acid either as the acid or as a salt, and a subsequent examination of its physical and chemical properties; by a combination of these methods; or by indirect methods.

##### 1. Examination; physical, chemical tests.

The methods of isolating lactic acid both in a form of the acid and as lactates will be given in the discussion of quantitative determination. The lactates are usually obtained and examined for their water of crystallization, loss of weight upon ignition, and occasionally for melting points. Certain of the lactates may also be recognized by their form of crystalline structure.

##### 2. Separation of lactic acid.

Davis gives the following procedures for separation of lactic acid from mixtures of other acids:

From organic acids forming insoluble lead salts, by precipitating with neutral lead acetate, either with or without addition of



alcohol.\* The soluble lead lactate may be decomposed to lactic acid by addition of  $H_2S$ .

Lactic acid may be separated from acids whose Ba salts are insoluble in alcohol by saturating the acid mixture with  $BaCO_3$ , evaporating, and treating the residue with alcohol. The soluble barium lactate may be decomposed to free lactic acid by cautious treatment with dilute  $H_2SO_4$  and filtration.

The racemic form of lactic acid may be separated as zinc lactate from acids which would still remain after removal of those having alcohol insoluble barium salts: Saturate the acid mixture with  $ZnO$ ; evaporate to dryness and digest the residue with alcohol. The zinc lactate is insoluble and could be separated by filtration. This method cannot be used for active lactic acid as that zinc salt is soluble in alcohol. (Davis gives an approximate quantitative method based on above, for estimation of inactive lactic acid.)

### 3. Tests upon oxidation products.

A number of methods for detection of lactic acid are based on the recognition of acetaldehyde as an oxidation product of lactic acid. The specificity of many of them are conditioned by presence or absence of other substances, and a choice of method depends upon the system under examination for presence of lactic acid.

In Windisch' method the lactic acid is oxidized by chromic acid; oxidation product (acetaldehyde) is distilled into warm Nessler's

solution. The aldehyde is recognized by yellowish red precipitate or yellowish opalescence produced upon addition of lead salts. This test may be used in presence of formic, acetic, propionic, butyric, valeric, succinic, malic, citric and tartaric acids; but it requires absence of alcohol, ammonia, and sugar.

Croner and Cronheim's method is based on the conversion of acetaldehyde into iodoform and the subsequent conversion of the iodoform into phenyl isonitrile, very small amounts of which can be recognized by its odor. This test is valuable in presence of other substances capable of giving rise to iodoform under the conditions of the test.

} substitute  
Insert  
given on  
following sheet

Herzog proposes the following method: Neutralize the solution with  $\text{Ag}_2\text{CO}_3$ ; concentrate by boiling to precipitate the silver salt. Heat the precipitated salt in a test tube containing alcoholic solution of iodine; pass reaction product (acetaldehyde and  $\text{CO}_2$ ) into a second test tube, with a trace of water. The presence of the aldehyde is proven if, upon addition of a little sodium nitroprusside and piperidine, a blue color appears, which, upon addition of a trace of  $\text{NaOH}$ , turns violet, then blue, and finally yellow; the aldehyde may also be proven by guaiacol or with codein. Czapek advises proof of the aldehyde by use of strips of filter paper soaked first in 10% sodium nitroprusside and then in a 5% solution of piperazin; this gives a blue violet color which can be confused only with propionaldehyde.

Deniges test depends upon oxidation of lactic acid to acetaldehyde by heating with  $\text{H}_2\text{SO}_4$  and subsequent recognition of that substance: 0.2 c.c. of a solution of lactic acid (up to 2%) is heated with 2 c.c. of concentrated  $\text{H}_2\text{SO}_4$  at  $100^\circ$  for two minutes; cooled; a drop of alcoholic solution of guaiacol should give a rose red color, or orange red with codeine.

Thom<sup>a</sup>' test for lactic acid in gastric extracts is also based on oxidation of lactic acid; add a few drops of 30% chromic acid to the gastric extract; heat on a water bath. A reddish brown color is given by traces of lactic acid.

( Insert p.    )

Vournasos' method is based upon the conversion of lactic acid into iodoform by Iodine and KOH; the iodoform upon addition of methylamine is converted into the isonitrile compound, very small amounts of which can be detected by its odor. Croner and Cronheim's method is similar to that of Vournasos. Aniline is added in the place of methylamine; if iodoform is present, phenyl isonitrile is formed. These tests, of course, are valueless in the presence of other substances capable of giving rise to iodoform under the conditions of the test.

The test is said to be indifferent to butyric, acetic, and hydrochloric acids, and to acetone or alcohol.

#### 4. Other reaction tests.

In Uffelman's test for lactic acid the amethyst blue color of the reagent is changed to a canary yellow in the presence of 0.01% lactic acid. It is not a specific test; other organic acids and  $C_2H_5OH$  give similar reaction. This test seems to be little more than a colorimetric determination of  $(H^+)$ ; strong acids discharge the color entirely; "the color of the reagent is weakened in the presence of an acid reaction". It may be used in presence of phenol and salicylic acid. It is used in examination of stomach contents for lactic acid; several investigators used it to test for complete extraction in quantitative analysis of lactic acid.

The following procedure is used in Hopkins' thiophene reaction for the detection of lactic acid: Place about 5 c.c. of concentrated  $H_2SO_4$  in a test tube and add 1 drop of a saturated solution of  $CuSO_4$  (which serves as a catalyst). Introduce a few drops of the solution under examination; shake the tube well; immerse it in boiling water for one or two minutes. Remove the tube, cool under running water, add 2-3 drops of dilute alcoholic solution of thiophene ( $C_4H_4S$ ), replace the tube in boiling water. Rapid formation of bright cherry red color denotes lactic acid; the color may be made more permanent by cooling as soon as color is produced.

This test is said to be more specific than Uffelman's.

The following yttrium lactate test has been used by several German investigators: Extract the acidified solution; evaporate the ether extract. Neutralize with  $NH_4OH$  and add a little yttrium salt solution. The yttrium lactate precipitated is highly characteristic --- "strong doubly refracting Mikrosphaerite".

## 5. Microscopic tests.

The following microscopic tests are of value in the detection of small amounts of lactic acid. (They are also probably more specific than the ones given above.)

### Indirect method:(Behrens)

Acidify solution with  $H_2SO_4$  (dilute); heat at  $130^{\circ}C$ . The lactic acid is split into  $CH_3CHO$  and  $HCOOH$ . Both of these reaction products may then be proven by microscopic analysis, by separate tests.

This method requires the presence of 5 mg. of lactic acid.

### Cobalt lactate method:(Behrens)

Addition of cobalt nitrate to concentrated solutions of potassium or calcium lactate causes the formation of clusters of fine reddish needles of cobalt lactate (after several minutes). Addition of cobalt acetate to solutions of free lactic acid gives the same result.

Crystallization in dilute solutions requires evaporation in the air or in a desiccator.

Beside the needles, there are also formed slender prisms with right and oblique angled terminal faces. Their length, (up to 200 microns), and their color render them more easily recognized than the small zinc lactate crystals.

This test requires fairly concentrated solutions; crystallization is hindered by the presence of malic and butyric acids and other impurities.

This method is in general favor. It was used by Herzog in proof of lactic acid formation by his zymase.

### Cobalt-lead-lactate method:(Behrens)

Barium and lead hasten the formation of crystals in lactic acid solutions treated with cobalt acetate.



With barium acetate, similarly grouped but thicker crystals are obtained than by the use of cobalt acetate alone.

Lead acetate produces thin, almost colorless platelets which are not grouped in clusters. Outline; elliptical: form; generally straight six-sided platelets, (40-70 microns), with three sharp-pointed terminal projections: terminal angle of six-sided platelets; 90°. Polarization; weak, negative, fading out or effaced toward the longitudinal axis.

For this reaction also, other acids must be removed as far as possible.

Add lead acetate; concentrate the solution as far as possible, if it remains clear. Finally add a small crystal of lead acetate; this causes the formation of the elliptical platelets of the double salt.

#### Zinc lactate method:(Behrens)

Crystals are prepared as in the cobalt lactate method. Occasionally the use of alcohol is of service in bringing about crystallization, but it is not usually necessary.

Zinc lactate forms small colorless prisms and needles (20-50 microns). These crystals stand out in such poor relief from the surrounding liquid that oftentimes polarized light is required for their recognition. This, and the slow crystallization, which is greatly hindered by the presence of impurities, make this method of microscopic proof of lactic acid less advantageous than those of the cobalt compounds.

Partheil used this method, as follows: Convert lactic acid into barium lactate; transform this to zinc lactate by addition of a calculated amount of zinc sulphate. Filter and examine microscopically the crystals after the concentration of the filtrate.

For other microchemical details, see Behrens, *Centr. 1900, 2, 10*

## V. Quantitative Determination of Lactic Acid.

In quantitative determinations of lactic acid, the general principles of analytical chemistry are applied to the chemical and physical properties of lactic acid itself and of the system in which it exists.

### 1. Acidimetric methods --- in absence of other acids.

In cases where no other acid is present, lactic acid may be determined by simple acidimetric methods. Lactic acid is a monobasic acid,  $\text{HC}_3\text{H}_5\text{O}_3$ , and the amount of lactic acid equivalent to the titration reading would be determined by the mass relationship between  $\text{NaOH}$  or  $\text{KOH}$  and lactic acid. In the media of agricultural lactic acid fermentations such determinations are open to the usual errors introduced by the presence of other substances, etc. The complete absence of other acids than lactic acid must be assured.

### 2. Methods used in fermentation mixtures.

The quantitative analysis of lactic acid is seldom so simple, as the media of most lactic acid fermentations are very complex and other acids are usually present in at least small amounts. In some of these analyses, the

lactic acid is isolated as the acid or lactate by extraction, distillation or precipitation; in other procedures, the other acids are first removed by distillation. In case the lactic acid is to be isolated, it must be remembered that the total amount of lactic acid probably is not present as free lactic acid, especially when neutralizing substances are present in the medium. To free it from its combinations, it is necessary to introduce a relatively strong acid ( $\text{H}_2\text{SO}_4$  usually, sometimes  $\text{H}_3\text{PO}_4$ ).

Choice of method of preparation of mixtures for analysis, as well as choice of method of final analysis is dependent upon the system under examination. A discussion of factors to be considered will be given after the presentation of the methods proposed.

#### Palm's method:

In 1893 Palm found that a definite chemical compound, basic lead lactate ( $3 \text{ PbO} \cdot 2 \text{ C}_3\text{H}_5\text{O}_3$ ), is formed by adding an excess of alcoholic ammonia to a solution of lactic acid and basic lead acetate. Later, he proposed a quantitative method based upon the insolubility of this substance in alcohol. This method is known as the "Palm basic lead lactate method".

Procedure:- Acidify with  $\text{H}_2\text{SO}_4$ ; extract with ether; evaporate the extract to a sirupy consistency; treat with water. Filter; add lead acetate; filter from any precipitate, and then add an excess of the acetate. Introduce alcoholic  $\text{NH}_4\text{OH}$ . Lead lactate is thus freed from other substances. Filter; wash the precipitate in alcohol. Ignite the precipitate, and determine as  $\text{PbO}$ . ( $3 \text{ PbO} : 3 \text{ PbO} \cdot 2 \text{ C}_3\text{H}_5\text{O}_3 = 78.5$ )

Van Slyke and Bosworth used this method in analyses of lactic acid content of cheese. They found it not altogether satisfac-

tory, but considered it the most efficient method then available. As early as 1899, Dobriner criticized it as not giving quantitative results, and in most recent investigations it has been supplanted by one of the following methods.

#### Partheil's method:

In 1903 Partheil proposed a method based upon vaporisation of lactic acid with superheated steam at high temperatures.

Procedure:- 10 c.c. of liquid are placed in a distilling flask connected to condenser and receiver; the system is maintained at  $110^{\circ}$  in an air bath. Pass superheated steam into the distilling flask; collect 300-400 c.c. of the distillate. Boil the distillate with excess and known amount of  $n/1$  KOH. Titrate back with  $n/1$   $H_2SO_4$ . This gives data for the calculation of the lactic acid.

This method is simple and direct, but is open to the following criticism. Suzuki and Hart found that although the lactic acid can be distilled without decomposition, the distillation is likely to be incomplete unless a large amount of the distillate is collected (750 c.c. recommended); low temperatures (below  $130^{\circ}$ ) likewise give low results. They also found it impossible to make a quantitative separation of lactic acid from other "fixed" organic acids; hence this method is inapplicable to analysis of many agricultural lactic acid fermentation media, due to the presence of malic, succinic, oxalic, and similar "fixed" acids.

#### Zinc lactate method:

The "Zinc Lactate Method" proposed by Buchner and Meisenheimer is based upon the isolation and preparation of the zinc salt of lactic acid and the direct weighing of the prepared lactate.

The procedure involves the complete ether extraction of the acidified material with ether, evaporation of the ether, and precipitation of zinc lactate by the aid of  $ZnCO_3$ . After



purification, the zinc lactate is weighed directly and the lactic acid calculated. Slight modifications of this method have been proposed by many investigators,\* differing in details as to extraction period, precipitation, crystallization, drying of crystals, etc. Ray and Nedig used the following manipulation:

Reduce to small volume; extract with ether for 72 hours. Evaporate off the ether; dilute with water. Boil with an excess of  $\text{Ba(OH)}_2$ ; exactly neutralize with  $\text{H}_2\text{SO}_4$ ; filter off the  $\text{BaSO}_4$ . Add  $\text{ZnSO}_4$ , avoiding an excess. Again remove the  $\text{BaSO}_4$  and evaporate to small volume on a water bath. As soon as zinc crystals are indicated, place the solution at a constant temperature (45° C.). Filter the crystals through a Gooch <sup>crucible</sup> wash with a small volume of water, and dry at 100° C. Subject combined wash water and mother liquor to a second and third crystallization; treat crystals as before. Add weights of the three crops of crystals and the sum represents the total weight of anhydrous zinc lactate in the sample, from which the lactic acid content may be calculated.

Fred and his associates claim that, due to the difficulty of preparing and removing all the zinc salt from solution, the zinc lactate method tends to give low results of lactic acid.

However, the zinc lactate method has many advantages; it possesses the accuracy of direct gravimetric analyses; as a check, the weighed anhydrous lactates may be recrystallized and examined for water of crystallization, or ignited for zinc oxide; it furnishes material

\* Fringsheim, 1910, Abh. Hdb. d. Bioch. Meth., Berlin, II, 29.

Kruse, 311.

Currie

Hertzog and Horth.

Harden (55b) --- precipitates by means of  $\text{CaCO}_3$ , converting lactic acid into calcium lactate.



by which optical properties of the lactic acid in the sample may be determined. This method is now used by most investigators in the determination of lactic acid content of agricultural lactic acid fermentation media.

Although lactic acid production by tissues is outside the scope of this paper, Wolf's method for quantitative estimation of lactic acid in blood and tissues\* is reported below, as it is probably adaptable to the analysis of many lactic acid fermentation media:

Presipitate proteins by Schenck's reagent (equal parts of 2% HCl and 5%  $\text{HgCl}_2$ ). Remove the mercury as sulphide and the hydrogen sulphide by aeration. Evaporate the acid solution to 10 c.c. in a Claisen flask at as low pressure as possible. Pour onto Adams' extraction paper; wash flask and deliver washings on second paper. While wet, strips are rolled up and extracted rapidly in Soxhlet extractor. After three or four hours, disconnect the flask and add 100 c.c. of water to the ether. Distillation of the ether leaves lactic acid in aqueous solution. Cool and filter.

Treat the filtrate with an excess of lead carbonate at  $100^\circ \text{C}$ . Cool and filter. Remove lead from the filtrate as sulphide, and the hydrogen sulphide by aeration. Then treat the filtrate with an excess of washed zinc carbonate at  $100^\circ \text{C}$ . Filter off the excess zinc carbonate and evaporate the filtrate to dryness. Weigh the zinc lactate.

\* No attempt is made to review the extensive literature on methods for determination of lactic acid in blood and tissues. A review of literature in this field may be found in the following references:

- Neuberg, 1911, "Der Harn", 245-254, 1170-1172.  
Emden, 1912, "Hdbuch. d. Bioch." V, 1255.  
Mondschein, 1912, Bioch. Zeit., XLII, 91, 105.  
Ishihara, 1913, " " L, 468.  
Yoshikawa, 1913, Zeit. f. Physiol. Chem.,  
LXXXVII, 382.

Determination of lactic acid in the presence of succinic acid.

Although volatile acids may be removed\* from samples being analyzed for lactic acid content, the separation of lactic acid from the "fixed" acids is more difficult. Succinic acid occurs with lactic acid in many agricultural products. For the analysis of these mixtures several methods are proposed, most of them based upon the relative solubility of different lactates and succinates.

One of these methods takes advantage of the relative insolubility of barium succinate in strong alcohol. Pringsheim claims this to give only 90-95% yield. Currie separated these two acids from cheese by an application of this method. Harden's method separates the two acids by means of the calcium salts.

Ayers and Rupp propose the following method for determination of content of each of these acids in a mixture of the two. It is an application of the relative solubilities of calcium lactate and succinate in 90% alcohol. Procedure:- Expel volatile acids; filter the remaining liquid; neutralize with NaOH; concentrate by evaporation. Acidify with  $H_3PO_4$  and extract with ether for 14 hours in a continuous extracting apparatus. Heat on steam bath for two hours the residue from ether extract with 100 c.c. of water and pulverized calcium carbonate. Shake occasionally. Filter the solution through an aluminum crucible; wash with hot water; dilute to 200 c.c. The calcium lactate and succinate are, of course, both still in solution. Determine as oxalate the calcium in a 50 c.c. aliquot. This represents the combined calcium lactate and succinate. From this data the combined lactic acid and succinic acid may be calculated. Then evaporate the remaining

\* See, however, p. V 2. g. for source of danger in the removal of volatile acids.

150 c.c. to dryness; dissolve the residue in 10 c.c. of hot water. When cool, add 90 c.c. of absolute alcohol; let the mixture stand two hours, shaking it occasionally. Filter; wash the precipitate with 90% alcohol. Free the filtrate from the alcohol by evaporation. Determine the calcium of the lactate as oxalate, and calculate the lactic acid. (If the content of lactic acid only is desired, the determination of the combined lactate and succinate may be omitted.)

In a recent investigation, Fred and associates determined lactic acid by converting it into its barium salt, which is then converted into barium sulphate. The sulphate is determined gravimetrically, from which the amount of lactic acid may be calculated. They obtained larger values of lactic acid with this method than with the zinc lactate method. This method is also adapted to analyses of mixtures of lactic acid and succinic acid.

#### Indirect methods of quantitative determination of lactic acid.

Besides these gravimetric and acidimetric direct methods of determination of lactic acid, many indirect methods have been employed. Most of these involve oxidation reactions and the gravimetric or volumetric determination of the oxidation product.

Dobriner proposed a method by which lactic acid is oxidized to oxalic acid; this oxidation product may then be determined by any of several procedures.

The determination of acetaldehyde, another oxidation product of lactic acid, is the basis of several quantitative methods. These methods differ principally in the procedure employed in the determination of the aldehyde. Davis points out that the difficulty of regulating the conditions determining a quantitative oxidation of lactic acid to acetaldehyde makes these methods less accurate than the direct zinc lactate method.

Partheil has proposed a gasometric method for determination of lactic acid in a mixture of volatile acids from the distillation of wine. It is based upon the formation of CO upon heating lactic acid with concentrated sulphuric acid. As Davis suggests, the accuracy of this method is questionable as Bistraychi and Siemeradski report that the reaction,  $(\text{CH}_3\text{CHOH.COOH} \rightarrow \text{CH}_3\text{CHO} + \text{H}_2\text{O} + \text{CO})$ , yields only 80-85% of the theoretical amount of CO. Other substances yielding CO must be absent or the test is valueless.

Factors to be considered in the quantitative determination of lactic acid.

The quantitative determination of lactic acid in the mixed systems of lactic acid fermentation is beset with difficulties which enter into many of the steps involved in these analyses. Wolf's careful and critical study of conditions governing the various steps involved in the determination of lactic acid in muscle tissue furnish<sup>es</sup> much of value, which should be incorporated in a study of determination of lactic acid in fermentation systems. His work involved a study of the following factors and individual steps: quantitative method for ultimate analysis of pure lactic acid; behavior of solutions of lactic acid on evaporation; extraction of lactic acid from aqueous solution; removal of proteins from solutions containing lactic acid.

For ultimate analysis of pure lactic acid he advises the zinc lactate method. He found that "given a pure solution of lactic acid in water, the acid may be quantitatively estimated by digesting with an excess of zinc carbonate." His results



with oxidation methods were less satisfactory, and he concludes that the zinc salt method is much better.

The evaporation of lactic acid is a step required in many procedures. It is well known that evaporation of lactic acid must never take place in alkaline systems. Wolf found also that it is "undesirable to concentrate solutions of lactic acid in open dishes on the water bath", and that it seemed "that all concentrations of lactic acid should take place at as low a temperature as possible, in a vacuum."

The extraction of lactic acid from the system is one of the most important factors to be considered, as all determinations by methods involving this process are conditioned by the completeness of the ether extraction. Due to the low coefficient of partition, "repeated extraction of the aqueous solution with large quantities of ether must be employed if removal of the acid is to be in any sense complete." Wolf found that this could be conveniently and quantitatively accomplished by use of Adams' paper.

Wolf believes removal of proteins before extraction of lactic acid is necessary, and that this is best accomplished by use of Schenck's reagent.

In some methods of preparing the fermentation mixture for analysis, the system is freed from volatile acids by distillation with steam. It has been shown that lactic acid is slightly volatile in steam, but authorities differ as to the degree of volatility. Jensen, Døx and Nedig, Waelde, and others, report that significant amounts of lactic acid pass over in steam distillates. Hart and Willaman report that lactic acid is but slightly volatile in steam at 100°. They claim that the amount passing over in distillation of silage is insignificant, being equivalent to not over 3-4 c.c. n/10 NaOH in 4 liters of distillate. The importance of the volatility of lactic acid is evident if preliminary procedures involve steps which may result in loss of lactic acid; its importance extends also to volatile acid determinations in systems containing relatively small amounts of volatile acids in presence of large amounts of lactic acid. (Partheil (1902) furnishes a review of the earlier conflicting reports on the volatility of lactic acid.)



Bellet (1913) had also made a report of a study of the conditions governing the quantitative determination of lactic acid. His conclusions are rather less convincing than those of Wolf. Bellet believed the principal difficulties to be encountered, came under the following three heads: (1) rapid and complete precipitation of proteïne; (2) the extraction with ether; (3) the ultimate determination of the lactic acid. He advised (1) the use of Fatain-Dafau reagent for protein precipitation; (2) for the extraction of lactic acid: concentrating to a syrup upon a water bath, collecting it into paper shells, and extracting them three hours in a Soxhlet apparatus; (3) for the ultimate analysis, he advises an oxidation method: conversion of the lactic acid into acetaldehyde and the passage of the oxidation product into an alkaline silver solution; the amount of lactic acid being calculated by the amount of reduced silver. While Bellet claims this method to be very accurate, direct determinations by the Zinc salts have been found by many good authorities (quoted above), to be more easily controlled and generally more accurate than any of the proposed indirect methods.

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### 3. Determination of "free" lactic acid. \*

The following three methods have been used by Van Slyke and Baker to determine the amount of free lactic acid in milk which has undergone lactic acid fermentation.

The lactic acid in a medium of lactic acid fermentation exists in three forms, as free lactic acid, unionized and ionized portions, and as combined lactic acid or lactate. The proportionate concentration of each of these forms will be changing during lactic acid fermentation, but, at any time, in accordance with the law of mass action, definite quantitative relations will exist between the amount of free but undissociated lactic acid, dissociated or ionized lactic acid and of combined lactic acid.

\* It has been mentioned that lactic acid may be present in the media of lactic acid fermentation, not only as free lactic acid but also in combined forms as lactates. The determination of the amount of free lactic acid present in the complex liquids of lactic acid fermentation is very difficult and any method is liable to error. Since knowledge of the presence of free lactic acid is important in many investigations, a brief statement of methods of its determination is given.

This definite quantitative relationship may be expressed by the equation:

$$\frac{a(b-x)}{x} = k_t$$

In this equation,

$a = (H^+)$  or ionized lactic acid.

$b =$  total amount of lactic acid.

$x =$  unionized or molecular lactic acid.

$k_t =$  a constant, varying with the temperature.

The following determination of the equation elements are required:

$a$  -- determined by hydrogen electrode  
(at  $25^{\circ}C.$ )

$b$  -- found by corrected titration.

$k_t$  -- determined to be a constant 0.00025  
in sour milk.

The amount of free lactic acid is determined by calculation from the above determinations.

$$x = \frac{ab}{a + 0.00025}$$

The second method is based upon the distribution coefficient of lactic acid between ether and water. This method gives reliable results only when no other ether soluble acid is present; it could not be used in cultures of lactic acid bacteria producing acetic acid.

Their "double electrometric titration" method involves determinations of the  $(H^+)$  of the medium after addition of measured portions of lactic acid and hydrochloric acid respectively. Measure the amounts of lactic acid and of hydrochloric acid that are required to bring the medium to the same  $(H^+)$ . The difference in the amounts of these two acids required is caused by the difference in the ionization of the two acids. Since the hydrochloric acid is practically all ionized and the lactic acid but slightly ionized, the difference in the amounts of the two acids that are required to produce the same  $(H^+)$  in two separate portions may be taken as a measure of the amount of free lactic acid in such media at that

particular (ii). By plotting the results of a series of such determinations, a chart may be prepared which will show the amount of free lactic acid present in this particular medium at any pH value.

Although these methods have been used only in milk investigations, it is very probable that by slight modification they could be adapted to other media and, if carefully controlled, would give means of determining the amount of free lactic acid present in many other agricultural products.

## VI. Stereochemistry of Lactic Acid.

### 1. History.

In the early day, lactic acid had been obtained from two general sources; from sour milk and fermented sugar solutions, and from muscle extracts. The comparative study of the lactic acid from these sources (usually termed at this time "fermentation" and "flesh" lactic acids) led to the development of the stereochemistry of lactic acid (and also played an important rôle in the development of the very fundamentals of stereochemistry itself).

In 1847, Liebig made a comparative study of the lactic acids obtained from muscle tissue and from sour milk. Altho' he observed a difference in the water content of the zinc and calcium salts of the two acids, Liebig believed that these differences might be due to the method by which he had prepared his crystals. As a result of his study, he announced that the two acids were probably identical.

Liebig apparently did not believe his results were conclusive, and turned over the remainder of the lactic acid he had obtained from flesh, to Englehart who made a further study of the two acids. He (1848) found that the questioned differences in the  $H_2O$  of crystallization of the salts of the two acids, were absolute and constant characters. In addition, Englehart found that the salts formed by



the two acids differed in solubility in water and in alcohol, in formation of crystals, and in course of dehydration. He believed that these differences precluded the possibility of the two acids being identical and termed the lactic acid from flesh, the "a" acid, and the lactic acid from sugars, the "b" acid.

Engelhart, however, made the mistake of interpreting the differences between the two acids, as due to the fact that the lactic acid from flesh or the "a" acid was monobasic, and the lactic acid from fermented sugars or the "b" acid was dibasic. (It will be remembered that Engelhart and Maddrell had reported just a year before, that the lactic acid obtained in the fermentation of sucrose was dibasic and capable of forming acid salts.)

Heintz (1848) followed with a report in which he corroborated the differences in the  $H_2O$  content of the salts of the two lactic acids. He also corrected Engelhart's assumption of differences in basicity of the two acids. The term "parasilchsäure" for the lactic acid from flesh was introduced by Heintz.

These studies of Liebig, Engelhart and Maddrell had succeeded in proving that the lactic acid obtained from muscle juice, and that (often) obtained in the fermentation of sugars, were not identical. The explanation of this phenomenon did not come until about twenty-five years later - a period during which many other questions on the nature of lactic acid were settled. (See "History of Lactic Acid".) During this period it was generally recognized that there were certain differences between the lactic acids obtained from fermented sugars and from muscle extracts. These

two acids were usually termed then, as "sarco", "flesh" or "pure" lactic acid, and "fermentation" lactic acid; in the later part of this period (after their optical investigation) also, as "active" and "inactive" lactic acids.

The first successful approach to an explanation of the phenomenon of the existence of the isomeric lactic acids is due to Wisnienius. He had made several noteworthy contributions to the knowledge of the lactic acids and of hydraacrylic acid, in the period mentioned in the preceding paragraph. Among other valuable steps in the development, it had been shown that of the two lactic acids only the one from muscle extract was optically active.

In 1873, Wisnienius carefully reviews his own work and that of others, comparing the properties of the active and inactive acids, their reactions and their salts. After an analysis of the possible differences between the two forms, he decides that their structural identity and dissimilar properties could be explained only by assuming a different arrangement of the atoms in space.

In support of his contention that the acids were structurally identical but geometrically isomeric, he presents the following:

"Ihre grosse Aehnlichkeit, ja ausgedehnte Gleichartigkeit in allen chemischen Eigenschaften, der leichte Uebergang der ersteren in die letzteren bei einfacher Erwärmung und ihre Verschiedenheit vorwiegend in optischer Beziehung sind von diesem Boden aus gleich erklärbar."

\*Van't Hoff admits that this idea of Wisclenius on the isomerism of "flesh" and <sup>"fermentation"</sup> lactic acids suggested to him the reflections leading to his theory of the asymmetric carbon atom.

According to this theory (which was itself suggested by phenomena associated with lactic acid fermentation) lactic acid or  $\alpha$ -hydroxy propionic acid, due to its possession of an asymmetric carbon atom, may appear in three forms: dextro-rotary form or dextro lactic acid, levo-rotary form or levo lactic acid, and inactive or racemic form, which is composed of equal amounts of the two active forms. All of these forms appear in lactic acid fermentation.

## 2. Distinction between active and racemic lactic acid.

The distinction between active and racemic lactic acid naturally consists in determining whether or not the acid is optically active.\* However, the determination of the kind of lactic acid present in media of lactic acid fermentation cannot be made merely by a deter-

\* Davis reports the following reaction test:- racemic lactic acid yields a deep blue liquid on addition of cupric sulphate, while active lactic acid is almost completely precipitated by that reagent.

mination of the optical characters of the medium. The presence of other optically active substances, the relatively weak specific rotation of active lactic acids, and the low concentration of lactic acid in the media of most lactic acid fermentations impose the employment of other procedures.

The lactic acid must first be isolated from the medium, usually by extraction (see "Methods of Quantitative Determination."). Solutions of lactic acid, however, are not well adapted to determinations of optical activity because of the low value of  $(\alpha)_D$ . Zinc or lithium salts of the lactic acid are usually prepared by one of the methods given.

The lactates of inactive and racemic lactic acids possess definite physical chemical properties which are of advantage in determining the stereochemical configuration of the acid from which they are derived. The lactates of active lactic acid exhibit higher specific rotatory powers than do the active forms of the acid itself. Hence, a determination of optical characters of a solution of carefully prepared zinc or lithium lactates furnishes a better means of determining whether the active or racemic form of lactic acid is present.



Other properties of the lactates lend themselves to such analyses: water of hydration; oxide content (and solubility). The properties of the zinc salt of the two forms follow:

	Racemic Form	Active Form
	of	of
	Lactic Acid	Lactic Acid
Composition formula	$(C_3H_5O_3)_2Zn \cdot 3H_2O$	$(C_3H_5O_3)_2Zn \cdot 2H_2O$
Water of hydration	18.17 %	12.9 %
Zinc oxide content	27.27 %	29.0 %
Solubility	53 parts water Insoluble in alcohol	17.5 parts water 1100 parts alcohol

The zinc lactates may be obtained by the method given and examined for the acid from which derived: determination of water of hydration by dessication, and of zinc oxide content by incineration. Even where optical determinations have been made, these determinations should be made as a check.

Scrupulous manipulation is required to avoid <sup>the</sup> following sources of error: the presence of lactic anhydride or other optically active substances; indecisive point of complete dehydration, due to impurity of the lactates, (especially evident in the case of active zinc lactate <sup>(C<sub>3</sub>H<sub>5</sub>O<sub>3</sub>)<sub>2</sub>Zn · 2H<sub>2</sub>O</sup>); loss of water upon drying of crystals at room temperature, (before dehydration) <sup>in the</sup> determination of water of hydration. The greater solubility of active zinc lactate may introduce error even before the final examination of the form of the lactate prepared. Because of its greater solubility, this form may be completely lost in the mother liquor during the repeated crystallizations for purification.\*

\* This error would occur in mixtures of racemic lactic acid and an excess of one of the active forms. By this error, media containing a small amount of one of the active forms would appear to contain only the racemic form.



The last named error is a particularly important one, as it occurs so early in the analysis. Its entrance into early analyses is probably largely responsible for the early idea that racemic lactic acid was the only form produced in lactic acid fermentation. (A belief which is reflected in the use of the term "fermentation" lactic acid for the inactive or dl-lactic acid.)

### 3. Distinction between dextro lactic acid and levo lactic acid.

After proof that the lactic acid is not the racemic form, it is necessary to determine whether it is the dextro or levo form.<sup>§</sup> The specific rotation of the acids themselves is comparatively low and the distinction between the two forms is made by examination of the rotatory character of the lactates. The lactates rotate polarized light in the opposite direction from their acids.

Heppe Gaylor and Araki have investigated the rotatory powers of different lactates and their results are still accepted. They found that the rotatory power of zinc, calcium, and lithium are dependent upon the concentration of their

<sup>§</sup> It will be shown later that any lactic acid fermentation must be a mixture of the racemic form and one of the active forms. In this case, weight of the lactic acid used, concentration of the solution, specific rotation value, actual angle of rotation observed, furnish data for the calculation of the per cent of active form present. (Vorsog and North, Irvin).

solutions, and that the value  $(\alpha)_D$  rises with the lowering of the concentration. For solutions containing the same concentration of lactic acid, this value is lowest in the calcium lactate and highest in the lithium salt. Because of its solubility, the ease with which it is dried, its beautiful crystallization and relatively strong specific rotation, they advise the use of the lithium lactate in the determination of the stereochemical configuration of lactic acid. The zinc lactates are used by many investigators because of the ease of obtaining these lactates and the accuracy of the quantitative method from which this material can be obtained.

Sources of error are also to be encountered here, among which is the presence of lactic anhydride. This substance is strongly levo-rotatory and might change the rotation of a solution of zinc lactate from dextro to levo. Salts of the anhydride possess opposite but definite rotatory powers.

The influence of the sources of errors mentioned above has probably had much to do with the widely divergent results reported in the literature of the stereochemistry of lactic acid fermentation, which is especially evident in the earlier investigations.

#### 4. Resolution of racemic lactic acid.

a. Pasteur's general methods of resolution.

The most marked difference in the physical and chemical properties of optically active isomerides is the rotation of the plane of polarized light in opposite directions. Hence,

\* There may be relatively slight differences in other properties, such as solubility and fusibility of their salts. E.g., dextro lactic acid and levo lactic acid are said to have about  $1^\circ \text{C.}$  difference in melting point; these properties, however, do not furnish a basis for separation.

# It is a general rule that anhydrides and lactones show a much higher specific rotation than do the acids from which they are derived. E. g., lactide with specific rotation  $(\alpha)_D$  of  $-86^\circ$  is obtained from lactic acid whose  $(\alpha)_D$  is  $+3^\circ$ . (Cohen)

the resolution of racemic compounds into their optically active components <sup>seldom</sup> can be accomplished by the usual methods based on differences in physical and chemical properties. The three general methods for accomplishing this resolution represent one of Pasteur's contributions to Science. With the development of modern chemistry, these general methods have of course been expanded and extended, especially in the domain of physical chemistry.

The following discussion of the resolution of lactic acid will be presented under the headings of the general methods of resolution introduced by Pasteur.

b. Mechanical separation or resolution by crystallization in enantiomorphous forms.

The first method depends upon the fact that racemates sometimes crystallize from solution in two forms, one corresponding to the dextro salt and the other to the levo salt. The crystal forms are mirror images and can be mechanically separated as Pasteur did in the case of sodium ammonium racemate (tartaric acid salt).

Irrespective of its rarity of occurrence, separation by crystallization as a means of resolution of racemic compounds is of special interest from a purely chemical standpoint, as it has an intimate bearing to many problems relating to chemical equilibrium. The relation of temperature to this method of resolution has been the subject of study by many physical chemists, who have shown that the formation of the enantiomorphs or of the original racemate crystals is often determined by an actual transition temperature. (Kendrick, XXI, 1749; Cohen, p. 75.)

This method has been applied to the resolution of the inactive salts of lactic acid by Purdie (1893), who separated inactive zinc ammonium lactate into its optically active components.

c. Method of formation of derivatives with optically active substances.

Pasteur's second method depended upon a difference in solubility of the salts formed by the union of optically active acids with optically active bases. If the optically active acids unite with an optically inactive base, as in the formation of a metallic salt, the internal structure of the molecules is unchanged, and the salts of the two forms still possess identical physical properties. However, when the optically active acids unite with optically active bases, the configuration of the salt molecules is changed, and they no longer possess identical physical chemical properties.

In the case of the resolution of racemic lactic acid, optically active alkaloids are made to unite with the two optically active components of the acid. The alkaloid salts formed differ in solubility and upon this basis the two forms may be resolved by fractional precipitation.

Purdie and Walker accomplished the resolution of racemic lactic acid by means of the strychnine salts. Junefleish's method of resolution of racemic lactic acid is based upon the quinine salts; Herzog found it gave poor results, and it does not seem to be used in recent investigations.

More recently, Irvines has resolved racemic lactic acid into the two optically active forms by means of the morphine salts. Morphine levo lactate crystallizes readily from dilute solutions, while the salt of the dextro acid is exceedingly soluble and came out only after several weeks in vacuum desiccator. He obtained an almost theoretical yield of



pure *levo* lactic acid and about 50% of *dextro* lactic acid. Procedure employed: neutralized an aqueous solution of racemic lactic acid with morphine; filtered. Upon cooling, the filtrate deposited the salt of the *levo* lactic acid; morphine *dextro* lactic acid remained in solution. The recrystallized salt was then converted into zinc lactate. This method is said to give good results and has been used in the majority of more recent investigations.

#### d. Biological method of resolution.

The third general method used by Pasteur is a biological method, and depends upon the "selective" action of certain microorganisms (or enzymes) upon one of the optically active components of a raceme. Since Pasteur's observation of this phenomenon with a *Penicillium* and the tartaric acids (1860), biological resolution has been reported in the case of a large number of racemic compounds. (See tables by Winther (1895), MacKenzie and Harden (1903), and Werner's "Lehrbuch der Stereochemie", p.63.) In the present century, it has also been extended to the resolution of certain racemes by enzyme solutions.)

The resolution of racemic lactic acid and racemic lactates by certain molds and bacteria has been reported by Lewkowitsch (1883), Linossier (1891), Frankland and MacGregor (1893), and MacKenzie and Harden (1903).

\*

The explanation of biological resolutions involves a number of questions of interest and importance from both physiological and chemical aspects. These can merely be mentioned here; a more complete discussion of these and related questions can be found later, in the report of "Stereochemical Lactic Acid Fermentation" and in the original literature cited.

\* See Addendum (1).



While the two active modifications of a substance seem to agree in physical and chemical properties, they may exhibit markedly different physiological properties.

That living microorganisms differ in their action upon enantiomorphs, would follow from the very fact of the "selective" assimilation which results in biological resolution. It is also interesting to recall that while one microorganism may consume the dextro modification of a raceme, another species may select the levo modification of the same raceme.

Several interesting examples are also reported of similar differences in the physiological action of enantiomorphs in the animal body. Piutti (1886) observed that d-asparagine has a sweet taste while the levo modification is insipid. Chabrie (1893) found that levo tartaric acid was twice as poisonous to guinea pigs as the dextro compound. Pictet and Retschy (1904) reported that l-nicotine is much more poisonous than d-nicotine. Examples of phenomena related to the above are seen in the power of the human body to assimilate and to excrete certain optically substances (Cohen).

\*

By many chemists, resolution of racemes by means of biological agents is explained in much the same way as resolution by alkaloids and other optically active compounds. In these explanations the microorganism (or enzyme) is considered as an asymmetric reagent. Winther (1895) presented an interpretation in which resolutions by alkaloids and by biological agents are alike dependent upon the stereochemical configuration of the raceme and of the resolving agent.

However, it is probable that, in the usual conception of the mechanism of biological

\*See Addendum (a)

APPENDIX TO VI. 4. d.

(1) The biological resolution of racemes is not limited to the action of microorganisms. The injection of dl-lactic acid (as the sodium salt) into rabbits is followed by the excretion of an excess of one of the active acids. (Hofe-Seyler and Araki), with CO-poisoned animals; Newbauer, with phosphorous-poisoned and normal animals; Parnas (1912), with normal animals.

(2) The experiments of Parnas (1912) furnish direct evidence of differences in the physiological effect of the different optical modifications of lactic acid. He reports that subcutaneous injections of the sodium salt of d-lactic acid into normal rabbits, proved harmless and were almost completely metabolized. Similar tests with the *levo* modification resulted in the excretion of a large portion of the *levo* in the urine; large doses (9.0 grams) of this enantiomorph proved toxic. Although an excess of the l-form was excreted in tests with the dl-lactic acid, Parnas found that more l-lactic acid is metabolized when injected as a constituent of the raceme than when the pure l-lactic acid was administered. Definite differences in the physiological action of enantiomers are applied in pharmacological practice. Among the important examples of the possession of different physiological properties by enantiomers, may be mentioned the *levo*-compound penicillin and the *dextro*-compound quinine.

resolution, the specificity of the action of certain microorganisms upon one of two enantiomers is exaggerated. The extension of Pasteur's resolution by living microorganisms to similar work with enzymes, has furnished further evidence that the "selective" action upon one of the active components is often merely a manifestation of differences in rates.

A presentation of investigations bearing on the mechanism of biological resolution will be given in the later part of the discussion of "Stereochemical Lactic Acid Fermentation".

#### 5. Racemization, or transformation of active lactic acids to the dl-form.

It has been shown above that inactive racemes may be resolved into their active components. It is also true that certain active substances may be transformed into the inactive raceme, by the conversion of one-half of the active material into its optical enantiomorph (Cohen, Molianari). This process is termed racemization.

Racemization may be effected by different methods. The operation of several of these has been observed in the case of lactic acid.

A rise in temperature is the most usual cause of racemization. The transformation of optically active lactic acid into the dl-form by means of heat, is reported by Wisclenius (1863), Herzog and North, Molianari and others. At 150°, active lactic acid is converted into the inactive lactide (Cohen).

The presence of foreign material frequently produces racemization (Cohen). Caustic potash is said to racemize optically active lactic acids (Cohen).

It is probable that, in many such cases, the presence of foreign substances tends to accelerate rather than to actually induce or produce racemization. (Indeed, Jungfleisch (1877) ascribed the racemizing action on tartaric acid by the oxides of iron and aluminium, to an actual catalytic action of these oxides). While racemization is most commonly effected by a rise in temperature, it is possible that often the increase in temperature is also simply an accelerating condition in the system.

The temperature at which some active substances are racemized, is quite low. With several substances the change has been observed to take place apparently spontaneously and at ordinary temperatures. This phenomenon is known as autoracemization.

Autoracemization of lactic acid has been reported by a number of investigators. Nef has observed such a transformation, which he explains upon the basis that dextro lactic acid is dissociated to a higher degree than is the levo form.

Salkowski (1909) reported that the d-lactic acid of Liebig's meat extract is transformed upon long standing to the dl-form. It is to be expected that these "autotransformations" would proceed at a much slower rate, than similar changes under higher temperature conditions.

MacKenzie (1906) has reported on the racemization of active lactic acid by chemical means.

Several interesting physical chemical explanations of the mechanism of racemization have been proposed. (Van't Hoff, 1877; Werner's "Lehrbuch der Stereochemie", p. 48; Erlenmeyer, 1919).



## B. Stereochemical Lactic Acid Fermentation.

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2. Lactic acids produced in lactic acid fermentation.

### II. Disposal of Early Explanations and Conceptions.

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1. Difficulty of explanation from static aspect.
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IX. Other Influences.

X. Question of Equilibrium Between Rates.

XI. Review of Factors Involved in the Mechanism of Biological Resolution.

## B. Stereochemical Lactic Acid Fermentation. (Kinds of Lactic Acid Formed)

### I. History.

#### 1. "Fermentation Lactic Acid" conception.

In the early days of our knowledge of lactic acid fermentation it was believed that the racemic modification was the only form of lactic acid produced in lactic acid fermentation. The influence of this early belief is still seen in the term, (coming from Liebig), "fermentation lactic acid", which is applied to the racemic lactic acid in many chemistry text books. It was soon shown, however, that the active forms appeared in the products of many lactic acid fermentations.

#### 2. Lactic acids produced in lactic acid fermentation.

In 1889, Nencki and Sieber(59c) reported that *M. acidii paralactici* produced dextro lactic acid, a form identical with the so-called "sarcos" lactic acid which Liebig had obtained from muscle extract. A year later, Schardinger(68) reported production of levo lactic acid in fermentation of sucrose by *Bac. acidii laevolactici*. A little later, Nencki(59a), Leichmann(49), Günther and Thierfelder(84), and others reported production of active forms by other bacteria. In 1900, Epstein(23) examined the forms of lactic acid produced by eleven species of lactic acid bacteria; nine of these produced racemic lactic acid; one, levo lactic acid, and another, dextro lactic acid.

Results of later investigations have established the fact that the product of lactic acid fermentation may be not only pure racemic lactic, pure dextro lactic acid, or pure levo lactic acid,

but there may also be mixtures of racemic lactic acid with an excess of either of its optically active components.

## II. Disposal of Early Explanations of Production of Active Lactic Acids.

To establish a better basis for a discussion of the conditions determining the stereochemistry of lactic acid fermentation, it is best to dispose of two assumptions made by the earlier investigators. Although at the time of their proposal they appeared well grounded, the results of later work make it impossible to accept them in the inclusive terms in which they were proposed.

1. Active forms not due only to biological resolution of racemic lactic acid.

Possibly largely under the influence of the early belief that racemic or "fermentation" lactic acid was always the final product in lactic acid fermentation, many authorities, (among them Lehmann and Neumann(48), Oppenheimer(60), and Czapek(14) ), explained in the following manner the appearance of optical forms among the products of lactic acid fermentation: The racemic form is always first produced (meaning, of course, that lactic acid bacteria always produce equal amounts of the dextro and levo forms); if no secondary reactions enter, that modification will be the form always detected in the final products of lactic acid fermentation.

The appearance of optically active forms in some lactic acid fermentations is due to the preferential combustion of one of the components of the raceme, leaving an excess of one of the forms. They strengthened these assumptions by the references given above under "biological resolution of racemic lactic acid into active forms". In cases where the racemic form persisted, it was assumed that the lactic acid bacteria present either did not attack the lactic acid produced or that they consumed equal amounts of the two optically active antipodes.

Benecke(6)<sup>and Mayer</sup> call attention to another fact that might, in some cases, be construed in favor of this hypothesis. In the experiments of Buchner and Meisenheimer\*, racemic lactic acid was the product of the lactic acid fermentation induced by the enzyme isolated from cells of *B. delbrückii*; while, with living cultures of this organism upon the same substrate, levo lactic acid is reported to be the product. Possibly here racemic lactic acid is first formed by the lactic acid bacteria zymase in the cell of the living *B. delbrückii* and subsequently the dextro form of the two raceme components is used up in some way by the life processes of the lactobacillus; this would result in the presence of optically active levo lactic acid in the final product.

Some lactic acid bacteria (probably rare with "true" lactic acid bacteria) may consume part of the lactic acid or of the lactates formed\*\* and some investigators, (Pere(63) and others), claim

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\* See p.      under "Enzymes of the lactic acid bacteria".

\*\* See "Reversal of Reaction".



that these lactics consume, in preference, a particular one of the optical antipodes of the racemic lactates. That this phenomenon can explain but few cases of the formation of active lactic acid in lactic acid fermentation has been experimentally demonstrated in a conclusive manner. Herzog and Horth(35b) show that it cannot account for many cases of active lactic acid production by the fact that active forms of lactic acid are the product of many lactic acid bacteria which give practically 100% yield of lactic acid from the sugar utilized.

Most authorities, while admitting that preferential combustion of one of the raceme components may at times play a role in formation of active acids in some lactic acid fermentations, believe it "highly improbable that the activity of fermentation lactic acid is due to the initial formation of racemic lactic acid and subsequent partial resolution into the two optical antipodes".

2. Product of one species not always pure dextro or pure levo lactic acid.

The second assumption was most strongly supported by Heinemann(33a) in his early work. In his investigations on *B. aerogenes* and *Strep. lacticus* in milk culture, he reached conclusions directly opposite to the theory just discussed. "Racemic lactic acid is the result of the formation of pure dextro lactic acid and pure levo lactic acid by at least two different species of micro-organisms. Racemic lactic acid is not known to be the product of one species only." It must be remembered that he had investigated but few strains and these only the most common members of the first two groups;

milk was the only medium tested. Obviously, this conclusion was based upon too little data to be applied as a general principle in lactic acid fermentation. Basing their conclusions on the specific nature of enzymes, MacKenzie and Harden(53c) advanced similar views from their work with several molds.

This extreme theory has also been disproven. Heinemann(33b) himself later found that some lactic acid bacteria always produce racemic lactic acid in milk, the same medium he had used in his former experiments. Moreover, it has been found that in many cases in which optically active lactic acid is produced, the acid is neither the pure dextro nor the pure levo form, but a mixture of racemic lactic acid and a comparatively small part of the total acid, one of the optical forms. This would mean that both dextro and levo lactic acids were produced in these fermentations, but one in excess.\*

The last two articles have been concerned with the disposal of biological resolution of racemic lactic acid as an absolute explanation of the presence of active lactic acids as products of fermentation, and with the establishment of the fact that all possible forms and combinations of forms or stereochemical configurations of lactic acid can be, and are produced in agricultural lactic acid fermentations. Discussion of the factors deciding or influencing their production follow.

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\* See references given under forms of lactic acid produced by lactobacilli, on following pages.

### III. Factors Determining the Modification of the Product.

#### 1. Opinions of authorities.

Although opinions vary as to the relative importance of different factors, many authorities believe that several factors enter into the determination of the kind of lactic acid that will be produced in lactic acid fermentation. Oppenheimer (60b) proposes the influencing factors to be the organism and the carbohydrate substrate; Duclaux(18), the organism, carbohydrate, and nitrogenous food in the medium; Kruse(47b), the organism, the properties of the medium, and conditions for growth; A. Mayer(54), in addition to factors named, the temperature and other influences.

Other authorities ascribe almost entire influence to the species of lactic involved.

#### 2. Probable interdependence of factors.

Probably all these factors are concerned in at least some cases. Some of them are probably much more important than others, but to determine their relative influence, all other variables must be eliminated. The failure to do this, together with possible errors in analysis of the acid product, probably accounts for many of the contradic-

tory results reported.

#### IV. Rôle of Species of Lactic Acid Bacteria.

##### 1. Review of investigations.

Meneki(59a) believed the species to be the determining factor independent of other conditions.

Slightly later investigations indicated that species is not an absolutely independent factor.

The results of Pere's(63) and others' experiments on *B. coli*, and those of Kayser(43) on a number of species, (as well as those of many other investigators), indicated that the same species can produce stereochemically different lactic acids, according to substrate and other conditions of growth.

The results of the investigations which followed, by Utz(73b), Thiele(72), Kozsai(46b), and others, are difficult to interpret here, as little of their work was done with pure cultures.

The investigations of Heinemann(33a), and of Hölling(37), indicate that in the fermentations examined by them the species is the independent determinant of the modification of the product of the fermentation.

Jensen's(40) analyses of the rotatory character of the acid produced by a large number of lactic acid bacteria lead him to believe that the species is usually the only factor involved. "As a rule, neither the carbon nor nitrogen sources affect the modification of the lactic acid. Those species, which in milk form pure dextro or levo lactic acid, will also in a nutritive broth always form dextro or levo lactic acid, whether the source of energy be alcohols, aldoses, ketoses, pentoses, hexoses or polysaccharides." "Those strains, which in milk form purely inactive lactic acid --- i.e., with like quantities of dextro and levo acid --- will, as a rule, also under other conditions maintain the equilibrium between the two acids." This, apparently, does not hold so true for those lactic



acid bacteria which produce racemic lactic acid with an excess of the optical form. "Strains which in milk form more of the one (optically active form) than the other will, under less favorable conditions, generally form the acid which they most easily produce." This change in the relative amounts of the two active forms, (which might change the mixture to pure racemic acid, or even to one of the active forms alone), may occur simply as a result of continued cultivation of the lactic, and without any loss in the total amount of acid produced.

## 2. Modifications usually produced by the four groups of lactic acid bacteria.

In spite of the influence of other factors, it is possible to make certain general statements as to the stereochemical form of lactic acid usually produced by certain lactic acid bacteria when cultivated in a definite medium. To do this it is best to make all other factors constant, with the species the only variable.

It is generally agreed that *Strep. lacticus* produces dextro lactic acid in milk,\* as found by Günther and Thierfelder(84), Leichmann(49), Hölling(37), and Heinemann(33a). Kruse(47b) gives results of other investigators, from which it is safe to state that <sup>the</sup> most common members of this group of lactic acid bacteria usually produce this modification.\*\* Jensen(40) gives the constant production of dextro lactic acid as a generic character of his

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\* The group statements in these paragraphs refer more particularly to lactic acid fermentations of milk and milk products, which are the most important agricultural lactic acid fermentation media. Advantage is often taken of these generalizations in analyses of these products to determine the lactic group concerned in their fermentation.

\*\* See references to Hennenberg(34), and Herzog and Horth(35), for contradiction, under role of substrate.



"Streptococcus" group of lactic acid bacteria.

The type species of the acid gas group has been shown by Hölling(37), Heinemann(33a), and others, to produce levo lactic acid in milk cultures. The weight of evidence indicates that, at least in the agricultural lactic acid fermentations of dairy products, the most common members of this group of lactic acid bacteria produce that form of lactic acid.

With the lactobacilli group, no general statement can be made. Many investigations, (Currie (13), Heinemann and Hefferan(33b), Hastings(31), White and Avery(81), Barthel(2b), Jensen(40), and others), have shown that different lactobacilli under like conditions may produce all forms and combinations of forms of lactic acid. It will be noted here that the products of many of the lactobacilli would fall under the lactic acid fermentations stated above by Jensen to be subject to stereochemical variation in case of same strain.

With the fourth group of lactic acid bacteria, little evidence can be presented, and it is impossible to make any general statement. It is probable that many members of this group would fall under Jensen's Betacoccus and Tetracoccus, which that investigator found to form levo lactic acid,

and exceptionally, racemic lactic acid and dextro lactic acid, respectively.

### 3. Probable importance of species as a factor.

The species stands out as the most important factor in the determination of the optical modification of the product of lactic acid fermentation. As stated above, several authorities consider it the only factor involved.

Jensen(40) considers the rotatory character of the lactic acid formed an important specific character, and uses it as a generic character in his classification. He concludes that "the modification of lactic acid . . . . depends entirely upon the species of bacteria," although it would seem from his own work that this inclusive statement should be limited to those strains producing pure dextro or pure levo forms.

Heinemann(33c), who has always been a strong advocate of this absolute influence of the species, states in his latest publication "one type of micro-organisms always produces the same modification of lactic acid without regard to changes in condition\* or environment."

In spite of the evident importance of the species or strain of micro-organism as a determinant of the stereochemical direction of lactic acid fermentation, it seems unwise to accept it as the only factor involved in all cases. It is more easy to

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\* This statement of Heinemann's does not agree with the finding by Jensen(40) of cases where the modification of lactic acid produced changed after continued cultivation of the organism, (this, however, not in cases where pure dextro or pure levo were first formed.)

believe that conditions which influence the life processes of lactic acid bacteria may project an influence upon the stereochemical configuration assumed by the chief product of their metabolism. With strains which produce only one of the active forms, these influences probably would not be sufficient to change the modification of their products. With strains producing both modifications, (either in equal amounts, giving a racemic product, or in unequal amounts, yielding an excess of one of the active forms), other influences seen, at times, to play a more or less important role.

#### V. Rôle of Substrate.

Most of the investigations of the stereochemistry of lactic acid fermentation have been concerned with the factors of species and substrate. Part of the facts relating to the role of the sugar fermented have been given above, from which it is evident that different lactic acid bacteria may produce different optical forms of lactic acid from the same substrate.

Investigations reporting that the same lactic acid bacteria may produce different optical forms of lactic acid by their action on different substrates are given below.

Keyser(43) carried out experiments with many species, in which the sugar present was the only variable factor. The results indicate that the same species, under these conditions, very often produce different forms of lactic acid from different sugars. Hennenberg(34) found that *Bact. lactis acid.* (Weigmann), which belongs to *Strep. lacticus* group, although producing dextro lactic acid from lactose, gave levo lactic acid from all other sugars tested. Pere(63) found the substrate to play a significant role and claimed that the substrates most easily fermented by *B. coli* yielded dextro lactic acid, while with those offering greater resistance, levo lactic acid was produced. Pottvin(64) also reports that the different sugars yield different lactic acids upon fermentation by the same species.

Emmerling(21) has compiled a table of results of many early investigations of the influence of the carbohydrate upon the stereochemistry of lactic acid fermentation. The results, however, are so variable that it is of doubtful value here.

Later workers are not so ready to accept the substrate as such an important factor.

Herzog and Horth(35b) carried out an extensive investigation with eight lactic acid bacteria upon a variety of sugars. They determined not only the optical character of the lactic acid, but also the per cent of total lactic acid which was in optically active form. Their results indicate that, although the same lactic may produce optically different lactic acids from different substrates, the difference is largely a quantitative one in the proportionate amounts of the two active forms. Six of the eight strains produced an excess of the same optical form, but even in these cases, a difference existed in the per cent of the total acid that was optically active, (i.e., in the relative proportions of the two active forms).

In the still more extensive work of Jensen (40), (mentioned above), a comparison of a large number of carbohydrates showed no difference in the optical modification produced from the different substrates.

Gayon and Dubourg (1901) found that the fermentation of different carbohydrates by their lactobacilli always yielded the same kind of lactic acid. This obtained even when the relative amounts of the various other products varied with the different substrates. (See "Other Products").



Most authorities, (Pottevin(64), Harden(29), Herzog and North(35b), Jensen(40), Heinemann(33), and others), are agreed that the stereochemical configuration of the substrate is without influence upon the optical modification of the product of lactic acid fermentation.

The above paragraphs, on the influence of the substrate upon the stereochemical direction of lactic acid fermentation, may be summarized as follows: (1) The action of the enzymes of different lactic acid bacteria upon the same substrate may yield different optical forms of lactic acid; (2) the action of the enzymes of the same micro-organism upon different substrates seems to yield only quantitative differences in the proportionate amount of the two optical forms produced, the substrate being without influence in case only one of the active forms is produced by the bacteria in question; (3) the influence of the substrate, in those cases where any is evidenced, is independent of its stereochemical configuration.

Later, reports will be given of the influence of environmental conditions upon the optical modification of the product of fermentations induced by action of enzymes of the same lactic acid bacteria upon the same substrate.



## VI. Relation of Enzyme and Substrate to the Modification of Lactic Acid Produced.

From a purely static point of view, it is rather difficult to bring the above phenomena into concurrence with the usual conception of the specific nature of enzyme action. From the standpoint of the "lock and key" theory, it would seem that, if the "lactic acid bacteria zymase" inside the cells of different lactic acid bacteria is the same enzyme, the action of lactic acid producing enzymes should always yield the same modification of lactic acid. That different forms of lactic acid might be produced from different substrates, would not be out of harmony with this conception. However, it has been established that the stereochemical configuration of the substrate itself has no definite influence upon the stereochemical configuration of the product of lactic acid fermentation.

"The subject needs attacking from the dynamic, rather than from the static, point of view, rates of reaction need more investigation than the fitting of locks and keys." (Bayliss)

Theories based on assumption of two enzymes.

From a more or less dynamic standpoint, but still largely under the influence of the stereochemically specific action of enzymes, Currie(13) suggests that there must be two enzymes concerned

in lactic acid fermentation, one of which produces dextro lactic acid and the other, levo lactic acid.

Although preferring the theory given later, Herzog and Horth(35b) suggest a similar theory of two enzymes as a possible explanation of the production of different modifications of lactic acid. If action of both these enzymes proceeds at the same speed, racemic lactic acid will result; if one proceeds at a greater velocity than the other, an excess of that form will be produced, the amount of excess optically active depending on the relative velocities; if the product is pure dextro or pure levo lactic acid, the enzyme producing the other modification was not present.

Jensen(40) also believes two enzymes are involved. "We must therefore presume that dextro and levo lactic acid are formed each by its own independent enzyme." He explains loss of ability to produce one of the active forms by bacteria, which at first produced unequal amounts of the two active forms, by loss of power to produce the corresponding enzyme.

The presence of two enzymes has never been proven and the following explanations are simpler, more easy to accept, and well in accord with the opinions of recognized authorities. These theories are not so strongly influenced by the conception of stereochemically specific enzymes.(i.e., up to the point of being fermented), and are upon a strictly dynamic basis. They assume the presence of but one enzyme, which forms both dextro lactic acid and levo lactic acid.

Harden's explanation upon basis of stereochemical configuration of enzyme.

In his attempt to explain the origin of optically active lactic acids in lactic acid fermentation by the acid gas group, Harden(29b) proposed the following theory. He points out that the rearrangement of the three center CHOH groups, from which lactic acid arises when glucose is fermented by *B. coli*, (see Harden's first equation under "Chemical Changes"), would yield an inactive acid, provided that the change were brought about by reagents containing only symmetric molecules. However, since the change is brought about in the pres-

ence of the asymmetric molecules of the enzyme, it would most probably be influenced so as to proceed entirely in one direction, or more rapidly in one direction than the other, and thus give rise to an active acid. The activity of the acid produced would then depend entirely upon the stereochemical configuration of the enzyme and be independent of the substrate, (up to the point of whether or not it is fermented by the enzyme).

This theory, while it could not be applied to Harden's later conception of the nature of the chemical reaction involved in this lactic acid fermentation, is very suggestive and probably had much to do with the development of the following theory.

Dynamic theory based on one enzyme, which produces both dextro and levo lactic acid.

Herzog and Horth(35b) propose the following somewhat similar, but more far-reaching theory. There is only one enzyme involved; it forms both dextro lactic acid and levo lactic acid; the rates of the two reactions may be different. The relative speeds at which the two modifications are formed will determine which modification is produced in excess; if both are produced at the same rate, racemic lactic acid will result; in some cases, the difference in speed of the catalytic direction may be so great as to result in formation of the maximum acidity of one optical modification, before an appreciable amount of the other antipode is produced. They strengthened this theory by other stereochemical fermentation studies of Herzog and Meier(35c), and by work of Bredig and Fajans(10a) on the stereochemistry of catalytic reactions. (In a later paper by Fajans(14b), still stronger evidence is presented in favor of such an explanation.)

It is perhaps best to accept this theory of the origin of different optical forms of lactic acid. From this standpoint, it may be assumed that the two optically active lactic acids may be produced at rates depending upon the conditions named

at the beginning of our discussion of stereochemical lactic acid fermentation. Bayliss'(57) conception of similar phenomena in other enzyme actions may be applied here with more or less safety. From this point of view and by a projection of Fajan's (1940) quantitative measurements of stereochemical directions in other catalytic reactions, there may be all degrees of difference in the rate of these two actions. At any time in the fermentation, the kind of lactic acid found will depend on the product of these two actions. At the end of the fermentation, a certain equilibrium will exist between the optical forms. The position of this equilibrium point will determine the proportionate amount of the two optically active forms; its position will be determined by the above conditions.

Just what the relative influence of the species, substrate, and other factors is upon the direction of the catalytic action, is impossible to state. The enzyme of the species is without doubt the most important; however, from Herzog and North's results it seems that the substrate may often yield a quantitative influence upon the relative yield of the two active forms. It is very possible, however, that the influence of other factors discussed below are not negligible, and may,



at times, assume significance in the determination of the direction of the catalytic action and of the point of equilibrium.

## VII. Role of Nitrogenous Substances.

As has been shown under "Physiology of lactic acid bacteria", the amount and character of the nitrogenous substances in the medium exert a profound influence upon lactic micro-organisms. According to some investigators, a part of this influence is sometimes reflected in the configuration of the lactic acid produced by the micro-organism.

The experiments of Kayser(43), Pere(63), Bischler and Blachstein(85), and others, suggested that an intimate relation may exist between the kind of lactic acid and the source of nitrogen offered to some lactic acid bacteria. By altering the amount and character of the nitrogen nourishment, different modifications of lactic acid were produced. Bertrand and Duchacek's (7b) experiments with *B. bulgaricus* also show a decided influence of slight change in the character of nitrogenous food in the medium. In milk, a mixture of racemic and dextro lactic acid was produced; in lactose broths, the racemic form alone.

On the other hand, other investigators report no difference in the form of lactic acid produced in lactic acid fermentation media containing different sources of nitrogen. Kozai(46) found substitution of ammonium salts or asparagin was without influence upon modification of lactic acid produced in his cultures. Harden(29a) observed in different effect of substitution of aspartic acid for peptone. Jensen(40) reports that all of the many strains studied by him yielded products stereochemically alike in media offering different sources of nitrogen. Milk as a medium gave same modification of lactic acid as different nutrient broths.



### VIII. Influence of Temperature.

Although Pere(63) and others have reported observations of a change in optical form of lactic acid produced by the same organism in lactic acid fermentations at different temperatures, the influence of temperature is probably slight, if any, in pure cultures.

In many mixed cultures its influence would be very decided. In these cases, it would be explained as a result of a change in the flora, due to the gaining of ascendancy in the medium by those species best fitted for life at that temperature. If these lactic acid bacteria produced a different form of lactic acid from that produced by those dominant when the system was at a lower temperature, there would be, of course, a change in the optical form of the product. The conflicting claims of many authors, (Kozai(46), Utz(74b), Thiele(72), and others), as to the form of lactic acid in naturally soured milk are probably due to a change in the dominant lactic group.\* Any other condition of the "fitness of the environment" would have a similar influence upon the stereochemistry of lactic acid fermentations induced by a mixed lactic flora.

In the view of the apparent relative instability of solutions of lactic acid, as shown by Nef(58), the influence of temperature upon the equilibrium point of a system containing both optical forms of lactic acid cannot be entirely ignored. However, it is probably rare that this factor manifests an appreciable change in the optical form of pure culture lactic acid fermentation.

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\* See "Temperature Relations of Different Groups of Lactic Acid Bacteria", "Role of Species", "Stereochemical Lactic Acid Fermentation", Introduction to "Microbial Associations in Lactic Acid Fermentation".

## IX. Other Influences.

Pottevin(64) claimed that any condition tending to slow growth or hindrance of the life processes of the lactics, such as lowering available nitrogen content of the medium, raising of temperature, addition of antiseptics, or even a substrate offering greater resistance to fermentation, favored the production of levo lactic acid.

Bischler and Dzierzowski(86) report on the influence of oxygen concentration upon modification of the lactic acid produced by *Staphylococcus aureus*. They claim that, under usual oxygen conditions, the organism produced racemic lactic acid; under other conditions, an excess of one of the optical components.

Jensen(40) gives examples of the influence of continued cultivation of lactic acid bacteria upon the optical modification of their products. *Lactobacilli*, which when freshly isolated yielded mixtures of dextro lactic acid, together with larger or smaller amounts of the levo form, were found after several years to produce only dextro lactic acid. This occurred without any loss in the total acid producing powers of the organism.

It is not safe to dismiss entirely the possibility of the influence of other conditions. It is within reason to suppose that the presence of other products of the life processes of the lactic acid bacteria, some of which undoubtedly are optically active, might sometimes impose a certain influence upon the direction of the catalytic action.

## X. Question of Equilibrium Between Rates.

The whole question of the configuration of lactic acid produced in any system seems to be largely a question of equilibrium between rates of production of the two optically active forms. The

final equilibrium point seems to be determined largely by the enzymes of the species and by the substrate, altho' it seems probable that at times it may be displaced to a certain extent by any change in the system. (This displacement would not be appreciable in the case of lactic acid bacteria which under usual conditions produce apparently only one of the optical modifications in significant amounts.)

#### XI. Review of Factors Involved in the Mechanism of Biological Resolution.

Many of the same factors are probably involved in the determination of the equilibrium point attained in systems in which biological resolution takes place, as in the case of stereochemical lactic acid fermentation. Largely for that reason, the following reports are given in this place. While none of these observations have been made in studies of the resolution of lactic acid, an intelligent understanding of the biological resolution of lactic acid requires at least a consideration of the factors which may condition, or even determine, the operation of those forces which are responsible for the resolution of any racemic mixture.

The principal object of the following paragraphs is to suggest that the biological resolution of lactic acid (or of other compounds) is probably also a question of differences in rates and of final equilibrium. Recent investigations present facts which tend to a less strictly defined course of the process of biological resolution of racemic mixtures; and which suggest that the course

of this process may be modified by changes (qualitative or quantitative) in the resolving system.

As long ago as 1893, Frankland and MacGregor reported an observation which was not in accord with the earlier conceptions of the stereochemical relations of enzymes and of living organisms.

They found that, while fresh cultures of their *B. ethaceticus* acted only upon the dextre salts of glyceric acid, the organism could be gradually induced (by cultivation in a solution of calcium glycerate) to assimilate the levo enantiomorph.

Other examples have been furnished by more recent investigations, which show that neither the action of enzymes nor of living organisms need be limited to an attack upon one of two enantiomorphs.

Mayliss (1915) reports that there are many cases known where living organisms consume preferably the one isomer, but upon its disappearance also attack the other. Dox and Nedig (1912) found that extracts of *Asp. niger* hydrolyze both A and B-methyl glucosides. Such phenomena are in keeping with the dynamic explanations of the direction of other stereochemical catalytic reactions, which have been cited in the discussion of the "Relation of Enzyme and Substrate to the Modification of Lactic Acid Produced" (especially that of Bajans (1910)).

In a consideration of the mechanism of the biological resolution of racemes, the effect of the



presence of other optically active substances is not a negligible factor.

Wienmeyer (1908) presents results which indicate that the presence of an optically active substance (in itself indifferent) can influence the possibility of the action of an optically active enzyme upon the appropriate component of a raceme.

In an analysis of the factors involved in the resolving of racemic mixtures in certain systems, it is necessary to include also the factors which are involved in racemization.

It will be recalled that both chemical and physical changes in a system can at least accelerate the racemization of certain substances. It must also be acknowledged that there is a possibility of racemization and resolution occurring in the same system, or at least that it may be possible for the one process to be induced or accelerated by changes brought about in the system by the action of the agent of the other.

Many of the unexplained problems of biological resolution are probably intimately related and possibly dependent upon the many still unsolved problems, which are grouped together under the term of steric hindrance.



C. AMOUNT OF LACTIC ACID FORMED.

I. Relation of Lactic Acid Production to Sugar Transformation.

1. Theoretical yield.

a. High sugar content of agricultural lactic acid fermentation media.

b. Mass relationship.

2. Actual yield.

a. Efficiency of sugar attack.

b. Factors conditioning efficiency of ratio of lactic acid carbohydrate  
(Species, carbohydrate, and secondary reactions).

II. Amount of Lactic Acid Formed.

(Data)

III. Factors Influencing Amount of Lactic Acid Formed.

1. Exhaustion of sugar.

2. Limitation of enzyme action.

a. Species

b. Substrate.

c. Neutralization.

d. Temperature.

e. Food.

3. Degree of completeness of the reaction.

## C. AMOUNT OF LACTIC ACID FORMED.

### I. Relation of Lactic Acid Production to Sugar Transformation.

#### 1. Theoretical yield.

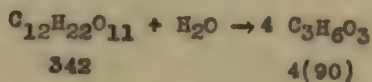
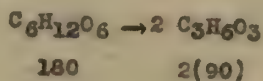
The theoretical yield of lactic acid from the fermentation of a certain mass of sugar requires, as is the case in any chemical reaction, that the reaction go to completion, that none of the sugar substrate be diverted into other reactions than those yielding lactic acid, and that none of the lactic acid produced enter into other, secondary reactions.

A measurement of the final concentration of the substrate will show whether or not all of the sugar has been used up by the life processes of the lactics, either in the reactions induced by their lactic acid fermentation enzyme or in sugar diverting reactions, induced by other of their enzymes. It will be shown below that very often part of the sugar does not enter into the reaction, or at least, that part of it remains unchanged at the end of the reaction. That this is the case in most agricultural

lactic acid fermentations is indicated by an application of the ratios given below to the comparatively high concentration of fermentable sugar in the media of these important agricultural fermentations.

A measurement of the final concentration of the lactic acid product, together with a comparison of the initial and final concentrations of the sugar, will give data showing the yield of lactic acid from a definite mass of the substrate. This will take care of the last two factors, --- the influence of sugar diverting reactions and secondary reactions upon the lactic acid.

According to the mass relationships of the equations given for "true" lactic acid fermentation, the theoretical yield of lactic acid in such fermentations of hexoses and dextroses should be as follows:



From the equations given above, the mass relationship between the amount of lactic acid produced from true lactic acid fermentation would be,

$$\frac{\text{gm. lactic acid produced}}{\text{gm. hexose fermented}} = 1;$$

$$\frac{\text{gm. lactic acid produced}}{\text{gm. disaccharose fermented}} = 1.003$$

## 2. Actual yield.

### a. Efficiency of sugar attack.

However, the reactions involved in the life processes of lactic acid bacteria produced different ratios between the amount of lactic acid produced and the amount of sugar removed, (as such), from, (or rather, transformed in), the system. This ratio may be termed the "fermentation efficiency factor" of that particular lactic acid fermentation. In lactic acid fermentation media, in which the reactions are brought about by "true lactic acid bacteria", the ratio is quite high and the amount of sugar diverted to other ends than lactic acid production is slight.

### b. Factors conditioning efficiency of ratio, lactic acid carbohydrate

This relation would seem to be dependent upon three factors:--- the strain of lactic micro-organism and the requirements of its life processes; the ease of fermentation of the substrate by the lactic acid bacteria zymase; diverting reactions, by which part of the substrate is transformed into products other than lactic acid, and secondary reactions, by which lactic acid already produced, (both acid and lactate), is transformed into other substrates.

The results of investigations of Kayser(50).

Leichmann(reference 80), Weigmann(80), and others, show that most members of the *Streptococcus lacticus* group produced from 0.93 to 0.985 gm. of lactic acid per gm. of sugar destroyed\*, or nearly the theoretical yield of lactic acid. Herzog and Horth's(35b) experiments show the distinct influence of the substrate upon the "fermentation efficiency factor". They found that the sum of mass of substrate unchanged plus lactic acid produced is different in lactic acid fermentation by the same strain upon different fermentable sugars.\*\*

The question of diverting reactions recalls the facts stated in the discussion of "Chemical Processes Involved", that apparently, the reactions involved in "true lactic acid fermentation" are much more complicated than those represented by the simple reaction formulas given above. Apparently, the life processes of even true lactic acid bacteria include other reactions upon the sugar, by which a certain small part of the total sugar transformed is diverted from the sphere

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\* Duclaux believes that at least 5% of destroyed sugar never appears in the lactic acid yield; this would prohibit yields of more than 95% of sugar fermented.

\*\* Influence of intermediate substances possibly plays a role in this phenomenon.



of action of the lactic acid producing enzyme. The products of these side reactions would include "unknown other products" and traces of such substances as aldehydes, alcohol, esters,\* and acetic acid.\*\* The influence of secondary reactions, (upon the lactic acid itself or upon lactates), is an evident one. Since they seldom are of appreciable moment in true lactic acid fermentation, this factor needs no further discussion than that already given under "Reversal of Reaction".

## II. Amount of Lactic Acid Formed.

The amount of lactic acid formed in different lactic acid fermentations is so variable and so dependent upon the conditions existing within, and surrounding the system, that nothing more will be presented at this place than the following data. These show the amount formed in the lactic acid fermentation of common laboratory media:

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\* Such products may account for "sour" taste and odor of milk which has undergone "true" lactic acid fermentation.

\*\* See "Acetic Acid" -- "Other Products" for references and discussion.

## Milk:-

### Per Cent of Lactic Acid

### Authority

**B. lactici acid** (*Strep. lacticus*)

0.9 to 1.25	Hastings(31a)
0.4 to 0.9 *	Lohnis(52)(ref.)
0.8 to 0.9 **	Van Slyke and Baker(75a)

The amount formed by some strains may be considerably less; weak strains are reported to be unable to produce lactic acid in sufficient amount to coagulate milk.

**Lactobacilli**

1.25 to 4.0	Hastings(31a)
0.80 to 3.0	Heinemann and Hefferan(33b)
Type A 2.70 to 3.7	White and
Type B 1.20 to 1.6	Avery(81)

## Sugar Broths:-

The amount formed in these media is almost always less than in milk, largely because of their smaller buffer content. See work of Rogers and Davis, and Rogers, Clark, and Davis (references under groups of lactic acid bacteria).

## Wash:-

**Organisms of fermentation industries.**

0.8 to 1.9	Hennenberg(61)
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This amount is increased by the addition of neutralizing substances in many cases.

## Commercial Preparation of Lactic Acid.

Milk residues, (whey or molasses of milk sugar), are used in the industrial preparation of lactic acid. These

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\* Original acidity of milk subtracted.

\*\* Recalculated from the author's data.

are fermented in the presence of neutralizing agents, giving a large yield of the acid (Molinari)

In laboratory preparation of lactic acid, a 20% solution of glucose is fermented in the presence of calcium carbonate until the sugar falls below 0.4%. (Davis)

### III. Factors Influencing Total Amount of Lactic Acid Produced.

Lactic acid is the product of a biochemical catalytic reaction. The final concentration of the product of any chemical reaction depends upon the initial concentration of the reacting substance and the degree of completeness of the reaction.

#### 1. Exhaustion of the substrate.

The concentration of fermentable sugar remaining unattacked in the systems of the most important agricultural lactic acid fermentations is considerably higher than the final concentration of lactic acid produced. (Milk, e.g., initial concentration of sugar, 5%; final concentration, about 4%; final concentration of lactic acid, usually 1%). The amount of sugar diverted from the lactic acid producing reaction may be ignored in the case of "true lactic acid fermentation". If, then, in the lactic acid fermentation of milk, all of the sugar present entered into the reaction,

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a much larger amount of lactic acid would be produced than has been shown to be the case. From this, it is evident that the amount of lactic acid produced in most agricultural lactic acid fermentations is not limited by an exhaustion of the sugar. The elimination of the influence of the initial concentration of the substrate\* leaves the degree of completeness of the reaction as the determining factor of the amount of lactic acid produced in the reactions involved in most agricultural lactic acid fermentations.

## 2. Limitation of enzyme action.

At any time during the fermentation, the degree of completeness of the reaction may be considered as the amount of sugar which has been attacked by the enzymes of the lactic acid bacteria up to that time, (the ordinate of the curve at time  $t$ , in graphs shown before), under the conditions existing in the system. In any lactic acid fermentation this would be a function of the correlation of the cumulative effect of the conditions prevailing in the system and surroundings to the biological properties

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\* This is not meant to be a definitely inclusive statement, for perhaps it is impossible to eliminate completely the factor of concentration of the substrate. It is probable, however, that its influence would appear only, (if at all), in the speed of lactic acid products. (See "Theoretical Progress").

of the lactic acid bacteria (as shown under Physiology).

As shown above, the reaction never goes to completion, the final end point is never reached in common agricultural lactic acid fermentations. Although fermentable sugar is still present in comparatively high concentration, the reaction yields no more lactic acid to the medium. The final position of this point is determined by the limitation of the catalytic power of the lactic acid bacteria zymase, which may or may not be at the same point as that of the other life processes of the lactic.

### 3. Degree of completeness of the reaction.

Hence, the final amount of lactic acid produced in any agricultural lactic acid fermentation will depend upon the position of the equilibrium point reached in the reaction producing lactic acid, which was discussed under "End Point of the Fermentation". This equilibrium point is reached in most lactic acid fermentations when much less than half the sugar is transformed. If means were provided, (see "neutralization"), to remove the reaction product from the system, the end point could be pushed far over to the right of the equation,  $C_6H_{12}O_6 \rightleftharpoons 2 C_3H_6O_3$ .

Such a reaction could not be expected to go to completion in the presence of its products, and especially when the reaction product inactivates the catalyst.



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I. OTHER PRODUCTS OF LACTIC ACID FERMENTATION.

## OTHER PRODUCTS OF LACTIC ACID FERMENTATION

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## OTHER PRODUCTS OF LACTIC ACID FERMENTATION

### I. General Discussion.

#### 1. Introduction.

A logical limitation of a complete discussion of products other than lactic acid is rendered very difficult by the indefinite boundaries of lactic acid fermentation itself. As mentioned before, there are many fermentations in which lactic acid is produced in minute quantities; in such cases, a discussion of "other products" is, of course, out of the question.

In the following discussion, an attempt is made to divide these products into general groups, from the standpoint of their relation to the metabolism and life of the lactic bacteria.\*

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\* The writer realizes the more or less arbitrary division which results. It seems, however, that, based as it is upon the fundamental physiological requirements of the lactic microorganisms, it offers a convenient, and not illogical, basis upon which to proceed in a specific consideration of the different products.



In the first group are placed those products which are essential to the growth of all micro-organisms; of importance to, and necessarily derived and produced during, the life of lactic micro-organisms in any medium. In media not offering a substrate for the lactic acid fermentation process itself, the lactic would be required to derive and produce them by action upon some other constituent of the system. In media, in which the lactic acid fermentation reaction could take place, the substrate of that reaction would furnish the greater part of the most important of these products; others, probably, would be derived in large part from other substances present in the medium.

In the second group are placed those substances recognized, if not as products of the same general metabolic process, at least as being derived from the same substrate as that which yielded the lactic acid. Most of these products are not directly concerned in the growth of true lactic acid bacteria, but merely appear as products of a substrate which yielded energy to the agent of the fermentation.

## 2. Products directly concerned in the metabolism of lactic acid bacteria.

In the first of the above mentioned groups, would be included the construction of cells, energy, and metabolic products of unknown origin and nature.

### a. Cell construction.

Although a small part of the substrate carbohydrate may be utilized as substance for cell structure, the lactic acid fermentation process itself does not produce material for the building of cells. The reactions by which the carbon portion of the "food for growth", (which portion is small itself), is prepared and utilized by the cell are reactions probably more or less distinct from that resulting in production of lactic acid.

That the portion of the substrate diverted to furnishing material for the above mentioned purpose is very small, has been shown by quantitative relations between the amount of substrate consumed and the amount of acid products recovered. It is probable that the amount is even smaller than has been suggested by the above relation, due to the difficulty of a quantitative recovery of all of the products, (especially of lactic acid itself), and to the possibility of secondary reactions.

#### b. Energy.\*

Since lactic acid bacteria require, as do all organisms, "energy to actuate, as well as matter to form their mechanism", the energy yield of the lactic acid fermentation reaction assumes the greatest significance in the metabolism of lactic acid bacteria.

The action of the biological catalysts elaborated by these micro-organisms results in the reaction,  $C_6H_{12}O_6 \rightarrow 2 C_3H_6O_3 + 14.7 \text{ calories}$ . Hence, lactic acid and energy are the chief products of "true" lactic acid fermentation.

Lactic acid is utilized by so few lactic acid bacteria, it may be considered, from a purely physiological standpoint, as a substance left in the system as a by-product of the reaction, by which the micro-organism obtained its desired portion of energy. From this aspect, energy is indeed the fundamental product of lactic acid fermentation.

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\* A more complete discussion of the importance of the energy product has been given under "Energy Transformation", in the discussion of "Chemical Changes."

c. Metabolic products of unknown origin  
and nature.

It is impossible to state whether the unknown metabolic products, discussed under the "End Point of Lactic Acid Fermentation", are the result of the lactic acid fermentation process, or of reactions involved in other life processes. Possibly, certain substances remain as residues of carbohydrate substrates, as suggested by Brown; no definite evidence of this has ever been presented and it is doubtful if these substances are even of carbohydrate origin. From the probable close relation of at least some of these substances to toxins, it seems more probable that they are of protein nature and origin.

The "sour" flavor or odor of soured milk may possibly be due, at least in part, to other of these products, or to association of such substances and the acid. Van Slyke and Baker have shown that it is not due to the lactic acid itself. (See also "Esters".)

3. Products not directly concerned in metabolism  
of lactic acid bacteria.

This group of products includes a large number of substances, whose importance in many lactic acid fermentations makes them worthy of careful consideration in a study of these fermentations. In

many cases, these substances are produced in such large relative amounts that, in some of them, lactic acid may be regarded almost as a by-product. This becomes the case in many of the acid gas group fermentations. Although these products are usually produced in but small proportions in "true" lactic acid fermentation, the significance of their presence, even in small amounts, must be recognized.

Many substances may be included in this group of products: volatile acids, as acetic and formic; succinic acid; alcohol; gases, as carbon dioxide and hydrogen; and other products, as glycols, mannitol, esters, gums and mucilages.

#### 4. Factors.

Different fermentations show qualitative and quantitative differences in the yield of these products. The chief factors conditioning their production and relative proportions are the species of micro-organism, the substrate, oxygen concentration and other conditions in the fermentation system, as well as secondary reactions upon initial products.

##### a. Species and enzyme relations of the species.

The products of the fermentations induced by the different groups of lactic acid bacteria have been given before in the discussion under that heading. From that, it was evident that, even in the same system, different lactic acid bacteria produce



widely different products and these also in varying proportions. It is necessary here only to point out the general products of each group, and to pay particular attention to the factors conditioning the production of these substances in the various lactic acid fermentations.

In the case of the true lactic acid bacteria, especially with those of the lactic streptococci group, products other than lactic acid appear in but small amounts. In these cases, these other products may be considered, in a way, as by-products of the fermentation. With true lactic bacteria, Jensen, (1919), claims that the relative amounts, sometimes even the actual production, of these by-products are determined, to a certain extent, by the condition of the fermenting agent, such as time under cultivation. He claims that certain species which, when freshly isolated, produce considerable quantities of by-products, ("chiefly acetic acid and carbonic acid, at times also succinic acid; more rarely mannite and hydrogen"), "gradually lose" (upon cultivation) "partially or entirely, the power of forming by-products."

Although the products of the fourth group of lactic acid bacteria have not been so definitely determined, it is probable that lactic acid is not always predominant among the products of their fermentations, and that other products are formed in

considerable quantities.

It has long been known that the acid gas group of lactic acid bacteria produce large amounts of volatile acids and gases. Many of this group produce considerably less lactic acid than either formic or acetic acid. It has been mentioned before, (see "The Lactic Acid Bacteria"), that the difference existing in the products of fermentation by different members of this group is largely in quantitative relationship.

If we accept Harden and Penfold's, (1912), proposal of three simultaneous fermentations in case of acid gas lactic acid fermentation of glucose, the different quantities of the various products by different species, under like conditions, is explained as the result of differences in velocities of these reactions when induced by enzymes of different micro-organisms. From the standpoint of independent reactions, Gray (1918) has recently explained the appearance of different or varying proportions of products in acid gas fermentations as follows: "The nature of the fermentation products and the proportion in which these appear in the final analysis (of the fermentation mixture) will depend on the extent to which the various enzyme actions cooperate, which, in turn, depends on conditions, such as con-

centration of salts and temperature."

That the species of lactic is by no means the only factor is shown by the stringent limiting conditions that must be imposed when it is wished to differentiate between lactic acid bacteria by means of the products of their fermentations.

b. Substrate.

The substrate is an important factor in determination of the products of lactic acid fermentation, both qualitatively and quantitatively. This is particularly evident in the case of the acid gas group.

(1) Grimbert's table.

While so great a value of the substrate factor is unusual, the following table from Grimbert's (1895) work gives an example of the importance which the substrate may assume in the determination of the different products of some lactic acid fermentations.

Organism --- Bac. pneumoniae (Friedländer)\*

SUBSTRATE	P R O D U C T			
	Alcohol $C_2H_5OH$	Acetic Acid $CH_3COOH$	Lactic Acid $C_3H_6O_3$	Succinic Acid. $CH_2COOH$ $CH_2COOH$
Glucose	Trace	11.06 %	58.49 %	---
Galactose	7.66 %	16.60 %	53.33 %	---
Lactose**	15.00 %	19.53 %	Trace	30.73 %
Maltose**	Trace	35.53 %	Present Not determined separately	
Saccharose	Trace	29.53 %	43.6 % (as lactic acid) Not determined separately	
Arabinose	---	36.13 %	49.93 %	---
Xylose	6.90 %	23.40 %	Trace	19.90 %
Mannitol	11.40 %	10.60 %	36.63 %	---
Glycerol	10.00 %	11.82 %	27.32 %	---

(2) Composition and structure  
of substrate.

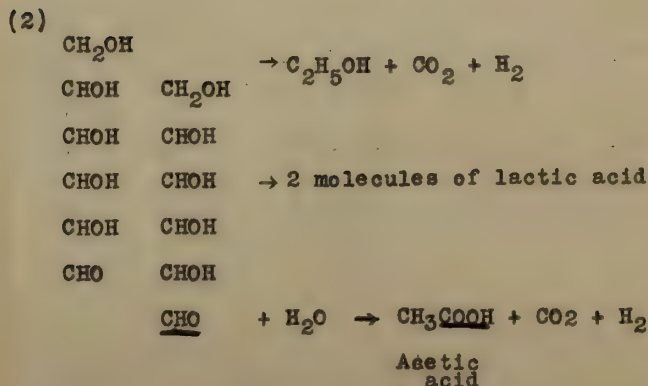
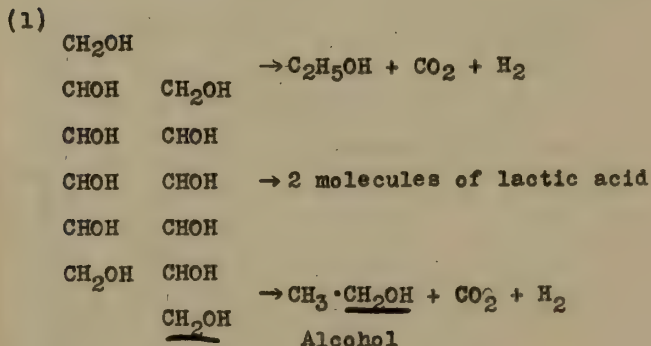
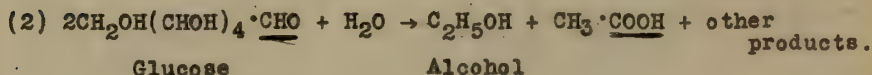
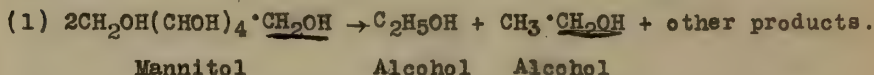
Harden and associates explained qualitative and quantitative differences in the products formed by members of the acid gas group as largely due to the composition and structure of the substrate.

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\* 'Lohnis' type species of first group of lactic acid bacteria.

\*\* Note that products of disaccharoses are different from those of the component monosaccharoses. This furnishes Kruse and others evidence for belief that hydrolysis does not always precede lactic acid fermentation of disaccharoses. (See enzymes, p. ).

Upon this basis, Harden and Walpole explain the differences in the relative yield of alcohol and acetic acid, when mannitol and glucose are fermented by *B. coli*: The greater yield of alcohol in the fermentation of mannitol is due to the presence of one more  $\text{CH}_2\text{OH}.\text{CHOH}$  group in a molecule of that substrate; the yield of alcohol, however, results in a corresponding decrease in acetic acid when compared to the products of glucose fermentation. The differences in these two reactions, as explained by the composition of the substrates, are evident in the following equations proposed by these authorities.



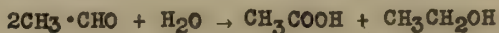


The true lactic acid bacteria, which do not produce other products to so great an extent as the above discussed group, also furnish examples of the role of the substrate in determining these products. Jensen points out that the fermentation of pentoses by true lactic acid bacteria must yield more by-products (as acetic acid) than the fermentation of hexoses. This is required by the composition of the substrate: the hexose molecule being split simply into two molecules of lactic acid, which, of course, is impossible in a similar intramolecular action upon five carbon sugars.

### (3) Intermediate substances.

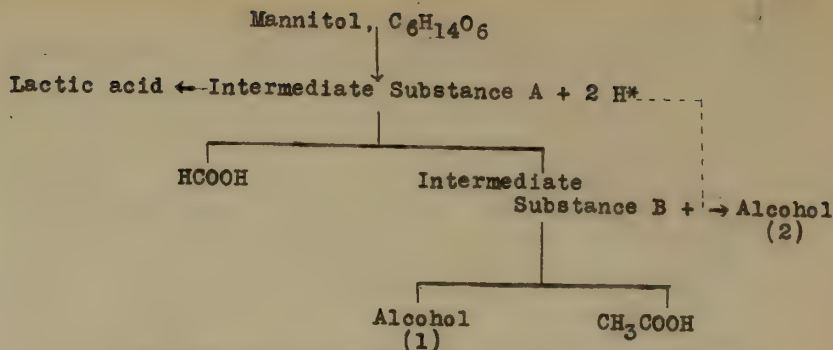
Grey (1914) explained the above differences in the relative production of alcohol and acetic acid in the fermentation of mannitol and glucose upon the basis of intermediate substances, as intimated in the discussion of that phase of "Chemical Changes Involved".

In the case of glucose, (see figures given on #I, 4, d, "Chemical Changes"), he believes Intermediate Substance B to be acetaldehyde. He suggests that the alcohol and acetic acid are derived in glucose fermentation by the following action on this intermediate substance.



This would give equimolecular proportions of alcohol and acetic acid, which is approximately the case in glucose fermentation.

He represents the mannitol reaction as follows:



The excess of alcohol, which results in the fermentation of mannitol, he attributes to the reducing action of the two atoms of hydrogen, (which are left after the formation of intermediate substance A from mannitol, but not from glucose), upon intermediate substance B. Thus, there are two reactions producing alcohol in the fermentation of mannitol, as compared to one yielding that substance in the fermentation of glucose: (1) formation of alcohol and acetic acid in equimolecular proportions from intermediate substance B (as in glucose fermentation); (2) in addition, a production of alcohol by the reduction of another molecule of intermediate substance B ( $CH_3CHO + 2 H \rightarrow CH_3CH_2OH$ ), by the reducing action of the two excess hydrogen atoms furnished by the mannitol molecule.

In his latest work, (1920), Grey emphasizes, in a more conclusive manner, the rôle of nascent hydrogen in the production of the different products. He is able to show that the relative production of succinic acid, acetic acid, and alcohol depends upon the degree of reduction brought about by hydrogen.

#### c. Oxygen concentration.

The effect of oxygen concentration upon the products of lactic acid fermentation may be considered from two standpoints; influence upon biological pro-

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\* Written as atomic H to show that it is intramolecular.

cesses of the lactics, or influence of secondary chemical reaction of oxygen upon the initial products after their formation.

From whatever aspect considered, experiments of Kayser and others already discussed show that, in most cases, under low oxygen concentration conditions, there is a relatively less amount of products other than lactic acid, than when the fermentation is carried on in systems of high oxygen concentration.

A recent report by Zoller and Clark on the production of volatile acids by dysentery bacilli emphasizes the importance of the influence of oxygen concentration in the relative production of these acids.

The quantitative relationship between the gases produced in lactic acid fermentation by the acid gas group has been established under anaerobic conditions, because of the influence of oxygen concentration. Keyes and Gillespie have shown that the relative proportion of gaseous products formed in fermentation of dextrose by *B. coli* varies under different oxygen concentrations.

#### d. Secondary reactions.

The influence of secondary reactions upon the relative proportions of these products is evident and important. It is of special moment in the production of acetic acid from lactic acid, the decomposition of formates, the reduction of accumulated mannitol, etc..

#### e. Other conditions in the environment.

The work of many authorities shows that still other environmental conditions at times play a definite rôle in determining the relative quantities of these products. Among these conditions are

temperature (Barthel) and hydrogen ion concentration of the system (Jensen).

## II. Specific Consideration of Important Products.

### 1. Succinic acid and other products not usually present in large amounts.

Succinic acid is formed to considerable extent even by some "true" lactic acid bacteria, as the lactobacilli. (See "Lactic Acid Bacteria"). This product assumes importance in the study of cheese.

The production of esters and other similar substances has been observed in the media of important agricultural lactic acid fermentations. Although usually representing but a small proportion of the products, their presence assumes importance in the flavors and odors contributed by small amounts of such substances. Their production may be due either to life processes of the lactic organism or to secondary reactions between other products of the lactic and constituents of the medium. No evidence has been presented to establish the actual origin of the substances or the substrate from which derived.

Many of the substances which might fall under the above heading are such common products of bacterial metabolism and are usually present in such small amounts that in many cases they may be considered more as products of other life processes

of lactic acid bacteria than of lactic acid fermentation itself.

The products discussed in the following pages, however, are quite abundant in lactic acid fermentations induced by lactic acid bacteria of the acid gas group. They probably are produced, at least in part, by the direct and more or less separate action of enzymes on the sugar within the cell, as explained under the discussion of "Chemistry of the Change", or possibly by still other reactions. Their relative proportion is decided by the conditions named above.

## 2. Acetic Acid.

### a. Production by the different groups of lactic acid bacteria.

The "true" lactic bacteria produce but small amounts of this product. However, even the *Strep. lacticus* group, whose close approach to a pure lactic acid fermentation lead earlier authorities to believe that lactic acid was the only acid produced in their fermentations, have been shown to produce small amounts of acetic acid. (See references under "Lactic Acid Bacteria"). The properties of this product make even these small amounts important in many agricultural lactic acid fermentations.

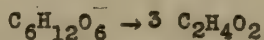
On the other hand, many organisms of the acid gas group produce much greater quantities of acetic than of lactic acid (Ayers and Rupp). In certain cases, the relative yield of acetic acid in some of these fermentations reaches such proportions that a discussion of these processes really



belongs to a study of acetic acid. Kruse calls such fermentations "anaerobic acetic acid fermentations."

b. Method of production.

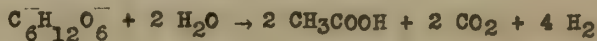
In "acid gas lactic acid fermentations", according to Harden and Penfold's proposed simultaneous reactions, acetic acid arises as a direct product of a certain enzyme acting upon a hexose molecule:



Kruse proposes the same equation for his "anaerobic acetic acid fermentation." From the above standpoint, the relative proportion of acetic acid would depend upon the speed of that reaction as compared to those induced by the competing enzymes upon the same substrate.

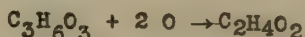
If Grey's theory of intermediate substances is accepted, (see "Chemical Changes"), the proportionate yield of acetic acid would depend upon the relative velocities of the competing reactions upon the intermediate substances.

Kruse proposes still other formulas for the production of this substance by direct enzymatic action upon glucose:

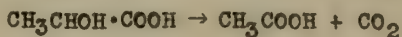


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\* Same as that first proposed by Harden (1901).

It has been shown that, in many cases, acetic acid is derived as a product of secondary reactions upon lactic acid. Kruse gives the following equation to represent the production of acetic acid by incomplete oxidation of lactic acid:



Peterson and Fred propose the following to represent the formation of acetic acid by secondary reactions upon lactic acid.



c. Factors conditioning its production.

It has been shown above that the relative production of acetic acid is dependent, to a large extent, upon the species acting as agent of the fermentation. The experiments of Kayser (1894) showed that other factors are also involved, such as the presence of neutralizing substances, substrate, oxygen concentration, and length of time of the fermentation. Duchakek (1904) has shown that a greater relative yield of acetic acid is obtained under aerobic conditions than in systems of low oxygen concentration. Unfavorable environmental conditions have been reported to result in greater yield of acetic acid in fermentations brought about by true lactic acid bacteria (Barthel, Jensen).

Examples of the production of acetic acid from secondary reactions upon lactic acid have been reported by a number of investigators (Kayser, Jensen, Fred and associates, and others). The relative yield in these cases would be dependent upon the relation between the products of these reactions at the time of analysis.

### 3. Alcohol.

#### a. Production.

Formation of alcohol is rare (Duclaux), or frequent, but present in small amounts (Kruse), in fermentations brought about by the "true" lactic bacteria. In the fermentations of the acid gas group, it is an almost constant product.

#### b. Factors determining its relative production.

In the determination of the relative proportion of this product, the same factors enter as those discussed above, under "Acetic Acid". Among these factors, the most important are species and substrate.

The importance of the substrate in particular reference to relative alcohol production in different fermentations has been discussed before. It is especially evident in the work of Harden and of Grey.

An example of the influence of the species is furnished by Harden and Walpole, who claim that *B. aerogenes* produces an excess of alcohol at the expense of that part of the glucose molecule which yields acetic and lactic acids when fermented by *B. coli*.

Fermentations in which alcohol is an important product, Kruse, (p.316), terms "alcoholic fermentation by bacteria", of which he furnishes an extensive discussion with references to the original literature.

#### 4. Formic acid.

##### a. Origin and production.

Formic acid is produced in considerable amounts in many of the mixed fermentations commonly included in the broad term lactic acid fermentation. With some members of the acid gas group, the amount of formic acid far exceeds that of lactic acid (Ayers and Rupp). The formic acid production by dysentery bacilli has been recently studied by Zoller and Clark, who suggest that the large yield of that acid by these bacteria may indicate a possible source for its commercial preparation.

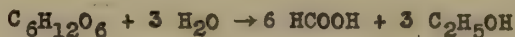
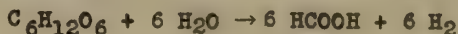
If Harden's simultaneous reactions\* are accepted for the acid gas fermentation, the large amounts of formic acid produced by certain of the

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\* See equations given under "Chemical Changes".

acid gas group can be explained by assigning a greater relative velocity to the formic acid producing reaction in their fermentations.

Kruse presents equations of still other reactions by which formic acid may arise in mixed acid fermentations.

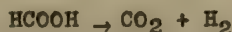


b. Relative amounts dependent upon equilibrium between rates.

The amount of formic acid present in the medium is not only dependent upon the determining conditions of its formation, but also upon secondary changes by which it is decomposed after its initial formation. In these cases, the quantity of formic acid present in the fermentation system at any time will be dependent upon the equilibrium then existing between the reactions producing it and those by which the acid and its salts are decomposed.

c. Secondary reactions by which formic acid is decomposed.

In many cases the formic acid is attacked by bacteria (which first produced it), according to the equation,



Many acid producing bacteria, which are closely related to the aerogenic members of the first group of lactic acid bacteria, are not able to decompose formic acid, and hence do not produce gas. This



relation is of diagnostic value in the differentiation of *B. typhosus* and *B. coli* (and other sub-groups of intestinal bacteria).

A large part of the formic acid produced usually exists in the medium as formates.

As shown under "Reversal of Reaction", many bacteria are able to attack these salts and transform them into carbonates and bicarbonates. It will be recalled that Ayers and Rupp explain the "methyl red test" upon the substitution of carbonic acid for formic acid as the result of these reactions.

Kruse gives a more complete discussion of the production of formic acid by bacteria. He also furnishes a review of the earlier work on these fermentations.

## 5. Carbon dioxide and hydrogen.

### a. Origin.

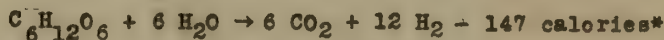
Carbon dioxide and hydrogen are the most frequent gaseous products of microbial metabolism. Carbon dioxide production need not in all cases be the result of acid destruction of carbohydrate, and it is possible that at least small quantities are formed by the life processes of all micro-organisms.

That small amounts of gas are either produced, or in some way liberated, in milk cultures of lactic streptococci, is a rather frequent laboratory observation. Probably this gas is carbon dioxide, as interpreted by Jensen (1919), who makes the following statement. "The majority of the true lactic acid bacteria, which do not develop any measurable quantity of gas, can, however --- likewise when in a state of particular vitality --- produce in milk so much carbonic acid that fine stripes appear in the curd."

Hydrogen, however, is a characteristic product of carbohydrate decomposition by bacteria and is found constantly in acid gas fermentation of sugars.

Both these gases are produced in the usual acid gas lactic acid fermentation; their formation there is probably due to the splitting of formic acid by the reaction given in the discussion of that product. Grey, (1920), however, is not so sure that carbon dioxide and hydrogen arise from the fermentation of pre-formed formic acid. He reserves a definite statement on this point until the publication of an investigation now in progress.

Kruse proposes several other reactions as the origin of these products. Among them is the equation,



\* Reactions yielding a complete oxidation product as  $\text{CO}_2$  are seldom endothermic. In this case it is due to the great amount of energy absorbed in the production of hydrogen. Such a reaction might as well be considered as a reduction of the complete oxidation product  $\text{H}_2\text{O}$ , which would, of course, be an endothermic reaction.

This is exactly the same thing as is seen in the energy relations of lactic acid fermentation; Here, there is both oxidation of the sugar and reduction of the water, just as in lactic acid fermentation there is oxidation of one carbon group and reduction of another; in this case, however, the amount of energy absorbed by the reduced substance overcomes that liberated by the oxidation of the other, resulting in an endothermic reaction.

Such an endothermic reaction could proceed only in the presence of other, strongly exothermic, reactions.

b. Ratio between these gases.

As these two gases are usually present together in the fermentation products, their quantitative relations have occasioned much research, resulting in the determination of this ratio for a large number of strains of acid gas bacteria (Rogers, Clark, and Davis).

Together with the methyl red test, the ratio between carbon dioxide and hydrogen furnishes the best basis upon which to divide the first group of lactic acid bacteria into sub-groups (Rogers, Clark, and Davis). When cultivated under strictly anaerobic conditions, the fermentation of glucose yields  $\frac{\text{CO}_2}{\text{H}}$  ratio of 1.06 for organisms of "low ratio" group (*B. coli*), while a higher value of  $\frac{\text{CO}_2}{\text{H}}$  is manifested in the fermentation products of the "high ratio" (*B. aerogenes*) group.

Although, with this group of bacteria, the gases usually occur together, as would be expected if they arose by splitting of fermentation acid, there are acid gas fermentations in which hydrogen is formed, but not carbon dioxide. The sub-group responsible for these fermentations, Rogers terms the "infinity ratio" group.

Fred, Peterson and Davenport found large yields of carbon dioxide among the products of their pentose fermenting organisms. Here they observed a striking example of the fallacy of gas measurements by the fermentation tube. Although as much as 27% of glucose was converted into carbon dioxide, no gas accumulation occurred in the closed arm of the tube.

For a complete discussion of origin and production of these gases, see the work of Harden and associates, and Rogers, Clark, and associates, in the case of the acid gas group; Kruse, for general bacterial production; Fred and associates, by pentose fermenters.

## 6. Glycols.

### a. Production.

In the discussion of the chemical changes involved in lactic acid fermentation it was stated that the reaction upon the sugar often yields other products than those appearing in the equations suggested. By analysis of the fermentation products of *B. coli* fermentation of glucose, Harden and Walpole found that the products given in equation 7 (under "Chemical Changes") do not account for all the carbon in the sugar, and that part of it entered into the production of a crude glycol.

### b. Oxidation of glycols by certain lactic acid bacteria.

The production of this substance deserves a brief discussion, as it furnishes the basis for one of the methods of distinguishing between the most common members of the organisms included in our first group of lactic acid bacteria.

In 1898 Voges and Proskauer had observed the formation of an eosin-like fluorescent color, by the addition of potassium hydroxide to sugar broth cultures of certain micro-organisms. They found the color change was not due to action of the alkali upon the sugar, but evidently to some action of the alkali upon the products of growth of the micro-organisms. This color reaction is known as



the Voges-Proskauer reaction, from the work of these men.

Later investigations have been concerned with the chemical and biological explanations of this phenomenon, and its adaptation to differentiation of coli-like bacteria. Harden and Walpole (1906) found that the crude glycol, found in fermentation products of *B. coli*, consisted largely of 2:3 butylene glycol ( $\text{CH}_3 \cdot \text{CHOH} \cdot \text{CHOH} \cdot \text{CH}_3$ ). This glycol may be oxidized to acetyl methyl carbinol,  $\text{CH}_3 \cdot \text{CHOH} \cdot \text{CO} \cdot \text{CH}_3$ , a volatile reducing substance, which, upon addition of potassium hydroxide in presence of peptone, produces an eosin-like color upon standing.

Even in the presence of peptone, the color reaction does not occur upon addition of potassium hydroxide to butylene glycol; under like conditions, it is produced with acetyl methyl carbinol, but not in the absence of peptone. Harden (1905) claimed that the color production was the result of a reaction between some constituent of peptone and diacetyl, which had been produced as the further oxidation product of the carbinol. In confirmation of this belief, Harden and Norris (1911) showed that, in the presence of strong potassium hydroxide solution, the reaction product of diacetyl and proteins gives the color and fluorescence of the Voges-Proskauer color reaction.

Both sub-groups produce the glycol, (in fact, it was in cultures of members of the Voges-Proskauer negative group that it was first found). The color reaction, however, is not given by the butylene glycol itself, but by its oxidation products. The aerogenes group oxidize the glycol to the compounds named above, and thus, in their cultures, a positive color reaction may be obtained; the *B. coli* group do not oxidize the glycol, and hence the color reaction does not take place upon addition of potassium hydroxide to cultures of this sub-group. That the production of the substance responsible for the color reaction is an oxidation process, is substantiated by the work of Walpole (1910), who showed that in the presence of oxygen *B. aerogenes* produced a larger yield of the carbinol.

As in the case of the "methyl red" and "gas ratio" tests, subdivision of the first group of lactic acid bacteria by means of the Voges-



Proskauer reaction is based upon differences in lactic metabolism --- in this case, manifested in the ability of but one sub-group to produce the carbinol by the oxidation of a glycol produced by both.

Harden and Norris, Levine, and others, have shown that acetyl methyl carbinol, which may be considered the basis of the Voges-Proskauer reaction, is a product of carbohydrate metabolism. It is not limited to fermentation of glucose; it is formed in the fermentation of a large number of sugars and alcohols; different lactics vary in their ability to produce the carbinol from different substrates.

The "Voges-Proskauer reaction" itself refers to the production of this substance in glucose media. Levine suggests the less specific term "carbinol test" for its production in other media.

The microbial production of acetylmethyl-carbinol is not limited to lactic acid bacteria. Grimbert (1901) reports the production of that substance by the action of *B. tartricus* upon glucose. Desmots (1904) has observed its formation by bacilli of the *B. mesentericus* group, by *B. subtilis* and by the so-called *Tyrothrix tenuis*. Mazé (1913) reports that acetylmethyl-carbinol is produced by the action of *Mycoderma aceti* upon lactic acid.

## 7. Mannitol.

The production of mannitol in systems in which lactic acid is also produced, has been reported thruout the history of lactic acid fermentation. Vaquelin (1807), and Pelouze(1833), observed the production of mannitol in the fermentation of vegetable juices. Many other of the older papers (Favre(1844), and others) report lactic acid and mannitol in the same fermentation system. Mannitol was regarded as one of the products of the "viscous" or "slimy" fermentations with which lactic acid fermentation was confused in the early day. In fact, the lactic acid fermentation of Pasteur included mannitol as a product usually accompanying lactic acid. However, these fermentations were of course, not induced by pure cultures and they in themselves furnish no direct evidence of the production of this substance by lactic acid bacteria.

In later work in which pure cultures were used, the production of mannitol by lactic acid bacteria has been observed by a number of investigators.

Much of the literature suggests that the production of mannitol is more frequent in the case of bacteria which prefer levulose to dextrose as a source of energy. (It is interesting to note that the earliest observations on the production of this alcohol were made in the examination of the products of fermentation of vegetable juices.)

Gayon and Dubourg (1894, 1901) and others report that their mannitol producers prefer levulose to dextrose.

The literature of mannitol production also furnishes many illustrations of the influence of the substrate upon the products of the fermentation. In some cases mannitol is produced in large amounts by the fermentation of fructose by organisms which do not produce significant amounts from glucose.

Beierjink (1901) reports that mannitol production is a character common to fructose fermentation by "aktiven" lactic acid bacteria. His "Aerobacter" group (acid gas group) produce considerable mannitol from fructose; the lactobacilli; large amounts; the lactococci; small amounts. Smit (1915) gives tables showing the relatively large amount of mannitol produced from fructose by a lactobacillus. Jensen (1919) states that "some few lactic acid bacteria can form--chiefly from levulose--a small quantity of mannite and hydrogen".

The relative amount of mannitol present in the system will vary in many cases during the course of the fermentation. Many lactic acid bacteria which produce mannitol, have also the ability to decompose it by secondary reactions which yield acid products, as lactic acid.

Pasteur found that in many cases, mannitol was not found among the final products of fermentation if chalk was present to neutralize the acid formed. He explained this as due to the combustion of mannitol by the ferment (or ferments).

In the careful work of Peterson and Fred, the significance of Pasteur's conjecture is evident. They found that considerable amounts of mannitol accumulated in the medium during the early stages of the fermentation of fructose by a lactobacillus. Later, this alcohol served as a substrate for acid fermentation, and gradually decreased in amount.

Further information on the production of mannitol,

the conditions influencing its relative production, the substrates from which it is derived, and equations suggesting the methods of its production can be found in the following references: Gayon and Dubourg (1904), Duclaux's discussion of Gayon and Dubourg's work, Smit (1915), and Peterson and Fred (1920).

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# Studies In Lactic Acid Fermentation

BY

JAMES M. NEILL

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PART II—III

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Part II.

"A STUDY OF THE CHARACTERS OF THE STREPTOCOCCI  
OF DAIRY LACTIC ACID FERMENTATIONS, WITH SPECIAL  
REFERENCE TO THE PRESENT STATUS OF THE SO-CALLED  
STREPTOCOCCUS LACTICUS GROUP."

### Cooperation of Mr. Roy C. Avery.

A part of the study to be reported in the following pages was carried on in conjunction with Mr. R. C. Avery. With his kind permission, I have included that work in Part II of this thesis. By the incorporation of this work, it has been possible to strengthen data obtained independently by additional and similar data on a larger number of strains of lactic streptococci.

I wish to take this occasion not only to acknowledge the cooperation of Mr. Avery in that part of the work in which he was actually associated, but also to express my appreciation of the extension of his interest to those parts of the investigation in which he was not an active associate.

## Part II.

A STUDY OF THE CHARACTERS OF THE STREPTOCOCCI OF DAIRY LACTIC ACID FERMENTATIONS, WITH SPECIAL REFERENCE TO THE PRESENT STATUS OF THE SO-CALLED STREPTOCOCCUS LACTICUS GROUP.

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## INTRODUCTION

The Streptococcus lacticus group originally proposed by Kruse in 1903 had almost unlimited boundaries and would include practically any nonpathogenic milk souring streptococcus. Today, the group is interpreted in much the same way by many authors. However, notwithstanding the fact that the so-called lactic group may be a most inclusive collection, there is a possibility that the present treatment is somewhat too indefinite to furnish a basis for the answer of a number of questions of biological and economic importance.

Its present status conditions the interpretation of the relation of the lactic type to the various other types recognized in proposed systems of streptococcal grouping. A more complete knowledge of the lactic group would aid most materially in the intelligent interpretation of the significance of streptococci in milk and milk products, and also should precede the assignment of the biological agency of important chemical changes in agricultural products. The establishment of the distribution and source of lactic streptococci and the question of whether this type is also responsible for the lactic acid fermentation of plant products must await the assignment of more definite boundary lines to the so-called Streptococcus lacticus group.

The present indefinite status of the lactic group is in no small part due to the reasons which have complicated the status of practically every group of streptococci. "Various authors in attempting to name or classify streptococci have fixed their attention upon different characters as criteria". (Brown, 1919)



For that reason, many of the frequent comparisons of Streptococcus lacticus with Streptococcus pyogenes could be interpreted only after an interpretation of the authors' conceptions of the meaning of the two terms.

The work of Sherman and Albus in the comparison of "pyogenic" strains presumably of udder origin with lactic strains obtained from soured commercial milk, was a distinct step in advance. The study of the streptococci of cheese by Evans (1918) furnishes still better evidence of the value of a cumulative characterization of the lactic group. By employing a number of characters, she was able to distinguish the true lactic from other types of streptococci which would be labeled Streptococcus lacticus by the usual, casual treatment.

Sherman and Albus proposed the following characteristics to distinguish the lactic type from the so-called Streptococcus pyogenes type; reduction of dyes, inability to ferment sucrose, ability to grow at 10° but not at 43° C. Evans (1918) has characterized the Streptococcus lacticus group as follows: characteristic reduction of litmus milk; production of about 0.12 g. of acetic acid per litre of skim milk; carbohydrates fermented in following order of availability: lactose, salicin, mannitol, and sucrose; formation of crystals in milk cultures by a large percentage of strains; decrease in true acidity of yeast peptone broth of initial pH 6.0 by a majority of strains. These and other characters which have been proposed in the differential study of the lactic group will be reviewed in the following pages.

## OBJECT OF INVESTIGATION

The present work is presented not as an attempt to characterize or bound the so-called Streptococcus lacticus group, but merely as a report on the characters exhibited by a number of strains which were present in large numbers in fermentation systems in which lactic streptococci are usually the dominant type.

## EXPERIMENTAL

### I. Selection and Isolation of Strains.

The number of strains studied in this investigation is not large, but special attention was given to their selection to prevent duplication.

The source of the strains is recorded below.

S	Cream
SK	Commercially pasteurized skim milk
G	Milk
C	Cream
W	Commercially pasteurized milk
M	Milk
MAC	Commercial starter
IN	Ice cream
X	Laboratory pasteurized milk
Z	Laboratory pasteurized milk
PD	Commercial starter
I	Milk
2	Commercial starter
3	Commercial starter
4	Milk
5	Oleomargarine
6	Milk
7	Butter
8	Butter
9	Milk

Strains S to PD were isolated in this laboratory with the following relations in mind:

Strains from milk and milk products: At the time of souring, the lactic type of streptococcus has become dominant in the natural flora of milk. For this reason, milk and cream samples were allowed to undergo natural lactic acid fermentation at room temperature. Plates were then poured from high dilutions to obtain the strains predominating in the various samples. The milk and cream were obtained from different producers since there is a possibility of certain strains becoming locally dominant in a

particular dairy. Only one strain was included from each producer or collecting station.

Strains from "Starters": The lactic type of streptococcus is commonly the microbial agent of commercial "starters". Due to the extensive use of these preparations in the controlled fermentation of milk and cream for the manufacture of butter, fermented milk drinks, and certain cheeses, a study of strains from these sources seemed particularly desirable. Strains were obtained by inoculating sterile milk with the "starter" and isolating the strain dominant in the fermented milk culture after incubation at room temperature.

Strains 1 to 9 were furnished as butter "starters" or as strains of lactic organisms, through the kindness of the Dairy and Bacteriology Departments of the following Agricultural Colleges: Vermont, Pennsylvania, Ohio, Michigan (2), Wisconsin, Kentucky, Florida and Oregon. The original sources of these strains are given as furnished by their contributors.\*

All strains were replated three times after the original isolation to insure their purity. Stock cultures were maintained by litmus milk cultures, which were placed in the ice-box after 12 hours preliminary incubation at 30° C. Unless noted otherwise, all characters were obtained by use of inocula of one tenth cc. from 12-hour broth cultures, which had been "invigorated" by four successive 12-hour transfers.

For comparative purposes, the following strains representing other types of streptococci were also included in some of the tests.

\*We wish to take this occasion to express our appreciation of their courtesy.

Human hemolytic strains: S67, S271, S84, S13, S125, S72, S2, S273, S70, S55, S23. These strains were furnished by Dr. O. T. Avery of the Rockefeller Institute for Medical Research. They represent a typical collection of hemolytic streptococci from human sources. Most of them were isolated from pathological conditions. The actual source of these strains and a further description of their characters are furnished by Avery and Cullen and by Dochez, Avery and Lancefield.

Hemolytic mastitis and udder strains: V1, V2, C53, C57, C59, C67, C69, M26. These strains were also furnished through the kindness of Dr. O. T. Avery. They represent a collection of hemolytic strains isolated from the udders of cows and from cases of mastitis. A further description of these strains is furnished by O. T. Avery and Cullen, by Jones, and by R. C. Avery.

Cheese strain: Strain MH. is included as a representative of the group of hemolytic "cheese" streptococci studied by R. C. Avery.

Sauerkraut strain: Strain K is included as a representative of a small number of strains isolated from sauerkraut in this laboratory.

## II. Morphology

### Previous reports:

The early descriptions of Leichmann and of Günther and Thierfelder describe the lactic acid organism as short rods with lanceolate or rounded ends, usually appearing in pairs or short



chains. Kruse, Hölling and Heinemann interpreted them as streptococci. The morphological resemblance of lactic streptococci to the pneumococcus was pointed out by Kruse, Hölling, and Saito.

Many attempts have been made to use as a differential character the tendency of lactic streptococci to form elongated rod-like cells. Similar attempts have also been made on the basis of length of chains. These distinctions are no longer considered of differential value. Evans (1918) states that Streptococcus lacticus cannot be distinguished by morphology.

#### Present observations:

Infusion broth cultures of the lactic strains exhibited chains, varying in length from diplococci to those with 12 to 16 cell members. In milk cultures, shorter chains and diplococci predominated.

### III. Reduction Phenomena in Litmus Milk.

#### Previous reports:

Heinemann (1906) in his studies on the relation of the lactic organisms to other streptococci, reported that "litmus milk is decolorized by Bacterium lactis acidi and all streptococci in the same typical manner". "The solid coagulum turns white leaving a pink ring at the top, which gradually extends toward the bottom". Apparently, this statement included the streptococci from pathological conditions.

Esten (1909) observed that litmus was completely reduced by the true lactic organism before coagulation occurred, (this sequence of coagulation and reduction is the opposite to that reported by Heinemann). He considered the reduction phenomenon exhibited in litmus milk cultures to be a valuable differential character for the lactic organism. Rogers and Dahlberg (1912) found that this test served as a means of distinguishing between strains from the saliva and from the udder of cows. Evans (1916) and Sherman and Albus (1918) have used this test to advantage in comparative studies of lactic and udder types. Hart, Hastings, Flint and Evans (1914), and Evans (1918) have found it of value in the differentiation of lactic streptococci from other types of streptococci found in cheese. Broadhurst (1915) did not find the behavior in litmus milk correlated with the origin of streptococci. Although many of the milk strains included in her study must have been true lactics, she does not mention reduction phenomena. Jensen (1919) does not consider the reduction of litmus milk of any value in the differentiation of lactic acid bacteria.

Salter (1921) has reported the behavior of a number of hemolytic human strains in litmus milk. One of the strains reduced the dye. He believed it a valuable character in distinctions between the more common streptococci of milk and pathogenic strains, although some pathogenic strains may not be differentiated by that means. Salter also describes a number of non-pathogenic hemolytic strains from milk which reduce litmus milk and closely resemble the so-called Streptococcus lacticus.

The characteristic behavior of litmus milk cultures of lactic streptococci is in general use among agricultural bacteriologists as a routine character in the determination of the Streptococcus lacticus group.

Present observations:

Procedure: Medium: Sterile skim milk, containing sufficient litmus to give the milk a robin egg blue color after sterilization. All of the strains of the various types were inoculated in this medium. Frequent observations were made to furnish records of sequence of reduction and coagulation and to avoid failure to observe possible, transient reduction.

Results: All of the so-called lactic strains rapidly and completely reduced the dye; reduction occurred before coagulation. None of the human or bovine cultures exhibited similar pictures, although incomplete reduction occurred with several of the human strains. The cheese hemolytic strain reduces litmus milk in exactly the same manner as do the lactic strains. The strain from sauerkraut gives only faint, if any reduction.

In conjunction with the 10° and 43° temperature tests, observations were made on the reduction pictures given by the lactics at these temperatures. At 43°, the same sequence of reduction was exhibited as at the usual incubation temperatures. At 10° all strains completely reduce the litmus as the first evidence of growth. However, due to the retardation of coagulation and the slow rate of growth, typical pictures are not always evidenced. The pink color, which returns, in many

cases extended to the bottom of the tube before coagulation occurred.

Although litmus milk culture is a valuable routine, preliminary test for lactic streptococci, it can not serve as an independent differential characteristic. The same reduction is also effected by certain members of other groups; it was exhibited by practically all strains of the hemolytic cheese group studied by R. C. Avery.

#### IV. Final H-Ion Concentration in Glucose Broth.

##### Previous reports:

Baehr (1910) reported that a larger amount of titratable acid was formed by lactic streptococci than by strains assigned to the Streptococcus pyogenes group. He believed this relation served as an aid in the differentiation of these two groups of streptococci. Similar statements are reported by several authors. Such observations are of course entirely dependent upon the various interpretations of the Streptococcus pyogenes group.

The introduction of H-ion concentration measurements have been of little value in the absolute differentiation of the lactic group. However, the pH values reached by the lactic streptococci in glucose broth have served to place them in the "high acid" group of Myers (1916). This character would also distinguish them from the "human" type of the so-called Streptococcus hemolyticus group (Avery and Cullen), but the final H-ion concentration of the lactic group varies within approximately the same zone as in the case of the "bovine" strains of the hemolytic group.

Present observations:

Procedure: Medium: Standard infusion broth, pH 7.2, containing one per cent glucose. Tests were made by the method described by Avery and Cullen (1919). H-ion concentrations were determined colorimetrically after 48 hours incubation at 37° C.

Results: The final H-ion concentration varied from pH 4.1 to 4.5. The values for the various strains are reported in the tabular summary.

In this medium, the final H-ion concentrations of the lactic cultures, as an independent character, merely places them in the large and heterogenous "high acid" group of Ayers. In the characterization of lactic streptococci, the value of H-ion concentration measurements seems to be that of a preliminary, but primary, differential character.

#### V. Behavior on Blood Agar.

Previous reports:

The confusion resulting from the differences in emphasis placed upon the various differential characters used in the characterization of streptococci, is especially evident in a review of the literature on the hemolytic ability of the lactic type of streptococci.

Müller (1906) found no difference in the hemolytic action of "pyogenic" streptococci and of streptococci from milk. However, his report cannot be used in the assignment of hemolysis to the lactic streptococci, as in the selection of his strains he probably ruled out most of the common lactic types upon



morphological grounds. Nieter (1907), Baehr (1910), Shippen (1914) report that the lactic streptococci exhibit no hemolysis. Saito (1912) reported that his lactic strains did not always show hemolysis; when present, it was "usually extended, diffuse, but often not complete". Puppel (1912) investigated a large number of strains from milk and compared them with those from human sources. He reports differences in the ability of the strains to hemolyze different kinds of blood. On rabbit's blood a number of the milk strains showed "strong" hemolysis. Puppel reviews the work of a number of authors and points out that, with the exception of Müller, these authorities agreed that the "milk streptococci" show no, or "only traces" of, hemolysis. Streptococcus lacticus is recorded as non-hemolytic on the blood plate, in von Lingelshein's summary (1912).

Ruediger (1912) reported that Streptococcus lacticus could be distinguished from Streptococcus pyogenes by the greenish discoloration of blood by the lactic colonies. Broadhurst (1915) reported that "green" or "green haze" discoloration was the only change produced on blood plates by twenty strains from milk (some of which probably were lactic streptococci).

Heinemann (1915) claims that after animal passage, two originally non-hemolytic strains of Streptococcus lacticus acquired the ability to hemolyze to some extent.

Davis (1916, 1918) reported that Streptococcus lacticus usually produces a green colorization on blood agar without appreciable hemolysis. He (1918) also reports on certain hemolytic strains from milk which are at least closely related to his non-hemolytic lactic strains. Salter (1921) has

also reported hemolytic strains from milk which seem to agree with the usual characterization of Streptococcus lacticus in all of the characters which he tested.

#### Present observations:

Procedure: Brown (1919) has emphasized the need of employing standard procedures in the study of the hemolytic action of streptococci on the blood plate. The conditions he advises were maintained in the present investigation.

0.6 cc. of defibrinated rabbit blood was added to tubes containing 12 cc. melted standard infusion agar, pH 7.4. Properly diluted suspensions were prepared from 12-hour broth cultures of each strain; the blood agar was inoculated and shaken; plates were poured and incubated in moist air at 37° C.

Macroscopic and microscopic observations were made at the end of 24 hours. A warm room was used for these observations and plates were returned to the incubator without delay. At the end of 48 hours incubation at 37° C., the plates were refrigerated at 10° C. for 48 hours. Examinations were then made.

#### Results:

##### Description of appearances on blood agar.

After incubation at 37° C.: Two strains, X and PD, exhibit the Beta type of hemolysis (Smith and Brown). Colonies were surrounded by a perfectly clear, colorless zone of hemolysis, after 18 to 24 hours incubation at 37° C. Most of the other lactic strains exhibited zones of greenish discoloration varying in extent. After 48 hours incubation, strains Z, 5 and 7

often produced very wide discolored zones around the surface colonies, at times simulating hemolysis unless examined carefully. Two of the lactic strains, W and S K, and the sauerkraut strain, never produced appreciable discolorization of the medium. Differences in behavior on blood agar seem to be exhibited by the surface and deep colonies of some strains.

After refrigeration: The above strains which had produced methemaglobin at 37° C., exhibited more or less clear zones surrounding a distinct inner ring of non-hemolyzed corpuscles next to the colony. These zones varied in area with the different strains.

The photographs shown in the following plates are offered as types of the different appearances observed.

Table I.

Behavior on Blood Agar Plates  
After Stated Periods of Incubation and Refrigeration.

Strain	24 hr. at 37° C.		48 hr. at 37° C.		48 hr. at 8° C.	
	Surface	Deep	Surface	Deep	Surface	Deep
S	Indiff.	Indiff.	Methem.	Methem.	R. Hem.	R. Hem.
SK	Indiff.	Indiff.	Indiff.	Indiff.	Indiff.	Indiff.
G.	Methem.	Methem.	Methem.	Methem.	R. Hem.	R. Hem.
C	Indiff.	Indiff.	Methem.	Methem.	R. Hem.	R. Hem.
W.	Indiff.	Indiff.	Indiff.	Indiff.	Indiff.	Indiff.
M	Indiff.	Indiff.	Methem.	Methem.	R. Hem.*	R. Hem.*
MAC	Indiff.	Methem.	Methem.	Methem.	Methem.	Methem.
X	Hem.	Hem.	Hem.	Hem.		
Z	Methem.	Methem.	Methem.	Methem.	R. Hem.	R. Hem.
PD	Hem.	Hem.	Hem.	Hem.		
IN	Indiff.	Indiff.	Indiff.	Methem.	Indiff.	Methem.
1	Indiff.	Indiff.	Indiff.	Methem.	Indiff.	R. Hem.*
2	Methem.	Methem.	Methem.	Methem.	R. Hem.	R. Hem.
3	Methem.	Methem.	Methem.	Methem.	R. Hem.	R. Hem.
4	Indiff.	Indiff.	Indiff.	Methem.	Indiff.	Methem.
5	Methem.	Methem.	Methem.	Methem.	R. Hem.	R. Hem.
6	Indiff.	Indiff.	Methem.	Methem.	R. Hem.	R. Hem.
7	Methem.	Methem.	Methem.	Methem.	R. Hem.	R. Hem.
8	Indiff.	Indiff.	Methem.	Methem.	R. Hem.	R. Hem.
9	Methem.	Methem.	Methem.	Methem.	R. Hem.	R. Hem.
K	Indiff.	Indiff.	Indiff.	Indiff.	Indiff.	Indiff.

Indiff. Indifferent

Methem. Methemaglobin Production

Hem. Hemolysis of the Beta Type (Smith and Brown)

R. Hem. Clear Zones after Refrigeration (Alpha Type.  
(Smith and Brown)

\* Clear Zones but Very Narrow

Methem. Very Wide Discolored Zones

It is evident in the above table that most of the lactic strains are methemaglobin producers. The two strains which exhibit the Beta type of hemolysis, have also been tested for their hemolytic titre in saline solution of washed rabbit blood cells according to the technique given in the U. S. War Manual No. 6. In this test, they produced complete hemolysis as rapidly as the human strains.

Tests of the behavior of all of the strains on blood agar have been made several times during the past year. While quantitative differences were observed in the methemaglobin production of various strains, in no case did a strain show hemolysis at one time and not at another. Tests made in which extract agar was used as the base, gave inconstant and difficultly interpreted results.

## VI. Volatile Acid Production

Previous reports:

Lactic acid bacteria of the Streptococcus lacticus type were formerly **supposed** to produce lactic acid alone as the product of the fermentation of sugars. (Leichmann) It has been shown, however, (Jensen (1904), Evans (1918), Hammer (1919), that small amounts of volatile acid are also produced in milk cultures of lactic streptococci. Hart, Hastings, Flint and Evans (1914), and Evans (1918) have used the relative amount of volatile acid produced in the fermentation of milk as a character of advantage in the differentiation of certain types of streptococci from the true lactic.



The possible advantages of the measurements of the actual products of the fermentation of sugars by streptococci as a means of describing streptococci are apparently unexplored.

The recognition of types of streptococci in cheese by Hart, Hastings, Flint and Evans (1914) and Evans (1918) is an indication of the differences in volatile acid production of different groups important in agricultural products. From the standpoint of the Streptococcus lacticus group as a member of the larger group of lactic acid bacteria, this is perhaps the most important and fundamental character. However, more should be known concerning the substrate of the volatile acid producing reaction before its use as a character in the grouping of lactic acid bacteria.

#### Present observations:

Procedure: The volatile acid production in skim milk was determined in the case of five lactic strains.

Flasks containing 500 cc. sterile skim milk were inoculated with 10 cc. of 12-hour milk cultures; analyses were made after 10 days incubation at 20° C. To obtain results comparable to those reported by Evans (1918), the same procedure was employed. Volatile acids were freed by addition of dilute phosphoric acid until culture was acid to Congo red. The cultures were distilled with steam until 2,000 cc. of distillate had been collected. The distillate was neutralized with barium hydroxide and evaporated to small volume. The barium salts were decomposed by addition of sulfuric acid. Volume was made up to 110 cc. and the mixture was distilled by the method of Duclaux. As comparative figures were all that was

desired at the time, the results were calculated and reported in terms of 0.1 N acetic acid.

Results: Jensen and Evans believe acetic acid represents at least most of the volatile acid production of lactic streptococci. All of the data obtained in this investigation do not agree with Duclaux's constants for acetic acid, which may be due to experimental error or to errors inherent in the method.

The cultures tested produced between 9.08 and 13.40 cc. 0.1 N acetic acid in 500 cc. skim milk. These strains showed a close agreement in volatile acid production with those studied by Evans (1918); who found about 0.12 g. acetic acid per liter in milk cultures of Streptococcus lacticus.

The results are recorded in the tabular summary. They are not presented as absolute values, but they serve to show that only small amounts of volatile acid are produced in milk cultures of lactic streptococci.

In spite of the unknown value of the relative volatile acid production in the grouping of streptococci, its importance in a consideration of the lactic streptococci cannot be over emphasized. The importance of such products of fermentation in butter and other dairy manufactures in which the lactics play a prominent part, warrants its further investigation.

## VII. Influence of Temperature Upon Growth.

### Previous reports:

Optimum temperature: Optimum temperature relations for growth should be based upon rates rather than upon final products. Such determinations are difficult to make, and there is considerable confusion between rates and final products in the literature concerning the optimum temperature for growth of the lactic streptococci. Most authorities agree on 30° as the approximate optimum temperature of the lactic organism.

Limiting temperatures: Leichmann (1896) reported that his lactic strain did not change milk in eight days at 9-12°C.; at 12-14° C. it curdled milk in six and one half days. Kruse (1903), Baehr (1910), Shippen (1914), Sherman and Albus (1918), and others have reported that Streptococcus lacticus has a lower minimum temperature than the so-called Streptococcus pyogenes group. Stowell and Hilliard (1912) came to the conclusion that temperature relations offer the most valuable differential character in distinguishing between the usual streptococci from milk and those from human throats.

The maximum temperature has also been used as a character of the lactic group. Leichmann (1896) observed scanty growth at 42° in milk cultures of his lactic strain; Sherman and Albus (1918) found most of their lactic strains did not grow in milk at 43° C.

### Present observations:

Procedure: Temperatures of 10° and 43°, which were used by Sherman and Albus in their study of lactic and "pyogenic"

udder strains, were chosen as test temperatures. 0.1 cc. of an 18-hour broth culture was introduced into 10 cc. of sterile litmus milk. The medium was brought to the test temperatures previous to inoculation, which were maintained during the procedure. Duplicates were run on each temperature series. The 45° series was incubated fifteen days; the 10° series, forty-two days. Two experiments were performed; one in February, 1920, and the other in March, 1921. The results are given in the following table.

For purposes of comparison, the behavior of the human hemolytic and the udder or mastitis hemolytic strains, to a temperature of 10°, are of interest. This was tested as follows: Inocula of 0.3 cc. of 18-hour broth cultures of each strain were introduced into duplicate tubes of glucose infusion broth. Observations of gross appearance were made weekly for seven weeks, after which time a colorimetric comparison of the pH value of the control and of the test cultures were made as a check on the presence or absence of growth.

Table II.

Growth of Different Types of Streptococci at 10° and at 43° C.

## Lactic Strains.

Strains Tested	10° C.	43° C.	
	1920 and 1921	Feb. 1920	March., 1921
S	growth	no growth	no growth
SK	"	" "	" "
G	"	" "	" "
C	"	" "	" "
W	"	" "	" "
M	"	" "	" "
MAC	"	" "	" "
IN	"	" "	growth
Z	"	growth	"
1	"	"	"
2	"	no growth	no growth
3	"	" "	growth
4	"	" "	no growth
5	"	" "	growth
6	"	" "	no growth
7	"	" "	growth
8	"	" "	no growth
9	"	" "	" "

## Sauerkraut Strain.

	1921	1921
K	growth	slight growth

## Human Hemolytic Strains.

12 strains no growth                      not tested

## Hemolytic Mastitis and Udder Strains.

8 strains no growth                      not tested

## Hemolytic Cheese Strain.

MH                      growth                      growth



Results: With the cultures tested, the 43° temperature test would seem to be of very limited value. The reason for the larger number of positive tests after a year's cultivation is difficult to explain.

The 10° C. temperature test would seem to be of value in differentiating certain types of streptococci from the lactic. All of the lactic milk strains grew at 10° C. After six weeks incubation, most of the strains had coagulated in milk. The cultures which had not produced sufficient changes in the milk to cause its coagulation at 10° C., coagulated within five minutes after being immersed in a 37° C. water bath.

None of the hemolytic human and mastitis strains produced any change in the glucose broth. Although all of the broth had reached a pH value of approximately 6.7, no difference in the H-ion concentration of the tests and controls could be detected.

While growth at low temperatures might possibly serve to distinguish the lactic type from most pathogenic strains, it would not serve as a differential character in comparative studies of other streptococci of more facultative temperature requirements. There are probably many types of streptococci possessing at least as low temperature requirements as that exhibited by the true lactic. As an example, many of the hemolytic streptococci from cheese studied by R. C. Avery grow readily at this temperature.

#### VIII. Ability to Survive Pasteurization.

##### Previous reports:

The heat resistance of streptococci is an exceedingly important character, although its value as a differential character of the lactics as a group is of limited application. The ability of different types of streptococci to survive the pasteurization of

milk is of great significance from economic and sanitary aspects. In the extensive and valuable literature which has contributed to the establishment of the conditions of the process, differences are evident in the heat resistance of different streptococci. It has been demonstrated that few, if any, pathogenic strains are able to survive thirty minutes heating in milk at  $62.8^{\circ}$  C. The lactic group vary among themselves in their ability to survive this heating process. Many of them, however, are able to survive in numbers sufficient to control the subsequent microbial changes in pasteurized milk. Salter (1921) has recently shown that in milk at  $60^{\circ}$  C., a higher thermal death rate is exhibited by hemolytic pathogenic strains than by "milk" strains.

Present observations:

Procedure: In our experiments, tests were simply made of the ability of the different strains to survive the temperature--time conditions of the usual pasteurization process, as in the older "thermal death point" determinations. It seemed that daily observations of the incubated tests would furnish means of distinguishing strains which survived the process in large numbers from those of which only a few cells survived. For the interpretation of the resistance of lactic strains to pasteurization, this is probably all that is necessary.

Tubes containing 10 cc. of sterile litmus milk were immersed in a water bath. When the milk attained the temperature of  $62.8^{\circ}$  C., 0.1 cc. of 18-hour broth cultures was added to duplicate tubes. Care was taken not to allow any of the culture to touch the sides of the tube during inoculation. Thermometer in control tube registered between  $62.5$  and  $63^{\circ}$  C. during the experiment. At the end of the heating period, the tests were plunged into running water at  $10^{\circ}$  C., and the tubes were then incubated at  $35^{\circ}$  C. Observations were made daily for one week.

Table III.

## Ability to Survive the Pasteurization Process.

Strains killed*		Strains surviving	
		Coagulated 24-60 hr.	Coagulated 84-120 hr.
(Lactic)		(Lactic)	(Lactic)
S	S 32 (Human)	SK	2
G	C67 (Mastitis)	PD	4
C	K (Sauerkraut)	Z	5
M		X	
MAC		l	
IN		Man. (Cheese)	
5			
6			
7			
8			
9			

\*Strains not surviving tests made both in 1920 and in 1921 are recorded in this group.

Results: The surviving strains may be arranged in two groups. The first group includes strains which seem to be able to survive pasteurization in large numbers. The second group includes strains which survive the process only in small numbers. It is probable that such lactic strains, if present in raw milk would be outgrown by more resistant strains after the pasteurization process. It is of interest to note that both of the hemolytic sour milk strains are included in the more resistant group.

The "human" and "bovine" strains included for comparison did not survive the process. (Salter found certain of his human strains survived 30 minutes at 60° C. in milk tests which had received very large inocula.) The "cheese" hemolytic strain survived the process, which is suggestive of the apparently general, resistant characteristics of the collection of strains of this type.

The value of the survival of this heating process as a differential character of any particular group of streptococci, is probably negligible. Strains vary within the different groups and there are other types of streptococci which may include as large a number of resistant strains as are found in the lactic group. From a practical standpoint, however, the heat resistance of the streptococci of milk assumes considerable moment in the determination of the types of streptococci which will control the subsequent biological changes in pasteurized dairy products, in cheeses which are heated during their manufacture, etc.

## IX. Pathogenicity.

Previous reports:

Baehr, Puppel, Saito and Gminder obtained negative results on tests of pathogenicity of Streptococcus lacticus to laboratory

animals. Hölling reported that mice are sometimes killed by injections of Streptococcus lacticus. Heinemann (1907, 1915) has reported an observation of increase of virulence of Streptococcus lacticus after repeated passage through rabbits.

The hemolytic milk strains of Davis (1918) and of Salter (1921) at least closely resemble the so-called Streptococci lacticus. Davis found that most of the strains gave negative results, although two of them seemed to show moderate pathogenic powers for rabbits. In tests made upon rabbits, Salter obtained "entirely negative results" from intravenous injections of his strains. The same results were obtained when mice were used. The effect of animal passage upon the virulence of these litmus milk reducing hemolytic strains, was tested by successive injections of typical strains into six mice. From the results of these experiments, Salter concluded that "it does not seem possible to render a strain of the hemolytic streptococci virulent by passage through mice".

#### Present work:

Procedure: 0.5 cc. of an 18-hour broth culture of each lactic strain was injected intraperitoneally, into white mice. (These tests were made soon after the cultures had been isolated from their sources, with the exception of those strains which were received from other laboratories).

Results: None of the mice exhibited any deviation from the normal control.

Although the question of virulence is always of first importance in a discussion of the biology of an organism, the value of inoculation of animals as a test of pathogenicity of streptococci is conditioned by many factors. Negative tests may be difficult to accept as final, but (as pointed out by Salter in his report of



similar experiments with hemolytic streptococci from milk), "when a large number of organisms of similar properties give constant results some conclusion may be warranted".

#### X. Sensitivity to Methylene Blue.

##### Previous reports:

Sherman and Albus (1918) reported that lactic streptococci reduced methylene blue in milk in a concentration of 0.005 per cent, and that udder strains of the "pyogenic" type failed to reduce the same concentration.

R. C. Avery, in studying the behavior of a large number of hemolytic streptococci from various sources, has shown that milk cultures containing 0.02 per cent concentration of this dye serve as a means of separating hemolytic strains into two more or less well defined groups. The non-hemolytic strains from various sources could not be easily separated upon this basis. A considerable number of udder strains reduced the dye in a concentration four times the strength of that used by Sherman and Albus. This suggests that a division between lactic and udder strains upon this basis is not clearly defined.

##### Present observations:

Procedure: Milk containing 0.02 per cent of Merck's medicinal methylene blue, received 0.1 cc. inocula of each of the lactic strains.

Results: The lactic strains are comparatively resistant to methylene blue, as complete reduction of the dye was effected by all of the strains of that type.

## XI. Fermentation of Carbohydrates.

1. Carbohydrates fermented.
2. Comparative availability of sucrose and lactose to sucrose fermenting lactic strains.

### Previous reports:

#### 1. Carbohydrates fermented:

Leichmann (1896) reported the fermentation of lactose, dextrose, maltose, and dextrin by the classical lactic organism.

Later authorities agree on the following fermentation reactions. Dextrose, lactose, and maltose are fermented by all strains; salicin and maltose seem next in order of availability; raffinose is seldom fermented and glycerol and inulin are almost never attacked.

There is considerable dispute over the fermentation of sucrose. Leichmann and Bazarewski (1900) report that it is not attacked. Jensen (1919) would not assign sucrose fermenting streptococci to the Streptococcus lacticus group. Sherman and Albus found but six out of fifty lactic strains fermented sucrose, and believed that failure to produce acid from this test substance was of value in the differentiation of the lactic from the "pyogenic" under types. The percentage of sucrose fermenting strains in their collection is much smaller than in those studied by Evans and other investigators. Jones, who found a number of sucrose fermenters in a collection of lactic strains, places considerable emphasis upon the ability of certain lactic strains to attack that sugar.

2. Comparative availability of sucrose and lactose to sucrose fermenting lactic strains:

It is well known that certain lactic acid bacteria prefer sucrose to lactose as a source of energy, as reports on sucrose

preferring strains are frequent in the literature of the fermentation of plant products. It seems that the relative availability of these two disaccharides in the case of sucrose fermenting lactic streptococci, is at least as important as the mere ability of these strains to form acid from that substrate. This relation has been tested in the second of the following experiments

Present work:

1. Carbohydrates fermented:

Procedure: Evans (1918) has shown that the final pH values reached by lactic streptococci varies with the strain and with different carbohydrates. Hence, determinations of the final H-ion concentrations in the various media did not seem to offer valuable means of characterizing these strains. For this reason, tests were simply made of the ability of the strains to produce sufficient acid to give Andrade indicator a definite magenta color.

Infusion broth, pH 7.2, was used as the base of the test media. This medium fulfilled Holman's requirement, as all strains grew well in it in the absence of a fermentable carbohydrate. One per cent of the test substance and one per cent of Andrade indicator were added to the basic medium.

2. Comparative availability of sucrose and lactose to sucrose fermenting lactic strains:

The availability of dextrose, lactose and sucrose as sources of energy to the sucrose fermenting lactic strains and to the sauerkraut strain, was tested by a comparison of the rate of acid production exhibited by equal inocula of these strains in fermentation systems differing only in the carbohydrate substrate.

Media: Standard infusion broth, pH 7.2, containing 1.1 per cent of Andrade indicator and of the test sugars. Medium was tubed

32.  
in 12 cc. portions in test tubes of uniform bore, and heated for three minutes at 120° C. No differences in the initial pH of the different sugar broths could be detected colorimetrically.

One cc. of 12-hour broth cultures of the test strains was introduced into 100 cc. sterile salt solution. One cc. of the dilution was inoculated into duplicate tubes of each of the test sugar broths. The test media were held at 37° C. throughout the manipulation and were then incubated at this temperature. Observations were made at 15 minute intervals and records made of the time required for the attainment of a distinct pink color. The color of the tests was compared with that of a strip of pink paper.

#### 1. Carbohydrates fermented:

Results: Dextrose, maltose and lactose were fermented by all strains; glycerol was not attacked by any of the strains tested. The results with the other test substances are recorded in the tabular summary.

The strain from sauerkraut fermented the following test substances; glucose, maltose, sucrose, raffinose, lactose and salicin.

With the exception of PD, the lactic strains exhibit the following order of availability of carbohydrates: lactose, salicin, mannitol and sucrose. This is the same order of availability as that given by Evans (1914, 1918) in her descriptions of the lactic group. A larger proportion of sucrose fermenters was found than that reported by Sherman and Albus. The fermentation of sucrose does not seem to be correlated with any other character, as may be seen in the tabular summary. This is also the case with the strains described by Evans (1918). This fact emphasizes the dangers

attending a division of the lactic group upon a single character, such as Jones' suggestion of Streptococcus lacticus I and Streptococcus lacticus II upon the basis of sucrose fermentation.



Table IV.

Comparative Availability of Sucrose and Lactose to  
 Sucrose Fermenting Lactic Strains:

Relative Rate of Acid Production in Various Sugars.

The rate of acid production is compared to  
 that of glucose as unity.

	Glucose	Lactose	Sucrose
K	1.00	.22	.92
W	1.00	.94	.94
1	1.00	.75	.56
2	1.00	.80	.62
3	1.00	.71	.55
C	1.00	.82	.53

## 2. Comparative availability of sucrose and lactose to sucrose fermenting lactic strains.

It is evident in the above table that none of the sucrose fermenting lactic strains exhibited a preference for sucrose. It is probable that the sauerkraut strain is a member of the large group of lactic acid bacteria which are particularly adapted to the fermentation of sucrose. It is believed that the value of the acid fermentation of sucrose as a character of the lactic group is limited, and that its only value would lie in the ruling out of strains which exhibit a striking preference for that substrate.

Differences in the rate of acid production from different carbohydrates have been observed frequently. This had been evident throughout all of our work on the carbohydrate fermentation reported before. In the case of several of the lactic mannitol fermenters, acid production was evident only after four to six days incubation, even with inocula of 0.1 cc. With the sauerkraut strain, the fermentation of lactose and salicin never occurred until after several days incubation. The rate of acid production from these two carbohydrates is strikingly different in the case of the lactic strains, in which group lactose and salicin were both fermented within 24 hours with the inocula used.

It is probable that, in certain cases, great differences in rates of acid production from different carbohydrates represents a distinction between substrates which serve as sources of energy for growth and those which are simply fermentable by enzymes (which may not <sup>be</sup> elaborated or liberated until later in the history of the culture). It would seem certain that the acid fermentation of salicin and lactose does not serve as a source of energy for the growth of this particular sauerkraut strain.

### XIII. Coagulation of Milk.

#### Previous reports:

A few authors have attempted to distinguish lactic streptococci from other streptococci by this character. Others have stated that "pyogenic" streptococci may be distinguished from the lactic type by the time required for coagulation of milk cultures of the two types. Jensen (1919), in fact, describes Streptococcus pyogenes as a type which is unable to curdle milk.

#### Present observations:

With the large and "invigorated" inocula used throughout our tests, all of the lactic streptococci studied here coagulated milk within 24 to 36 hours.

While it is true that most lactic streptococci curdle milk readily, this characteristic is by no means uncommon among hemolytic human and udder strains. Among the twelve hemolytic human strains studied in this investigation, seven curdled milk; all but one of the seven hemolytic udder strains also exhibited this character. Coagulation of milk is a comparatively frequent occurrence among the various types of streptococci.

Loss of ability of lactic streptococci to coagulate milk has been reported frequently. Several of our lactic strains failed to curdle milk when inocula from old cultures were used. However, no permanent loss of this character was observed. Although in some cases a large number of repeated transfers were required, all strains finally responded to successive sub-cultures, which were incubated at room temperature. It is possible that some lactic strains are temporarily weakened by continued cultivation at 37° C., although we have no experimental evidence definitely supporting this assumption.

Strain.	ose	Fermentation of carbohydrates.					
		Sali- cin.	Manni- tol.	Su- crose.	Raf- fin- ose.	In- ulin.	Gly- cer- ol.
X		+	-	-	-	-	-
PD	)	+	+	+	+	+	-
G		+	-	-	-	-	-
S		+	+	-	-	-	-
Sk		+	+	-	-	-	-
C		+	+	+	-	-	-
W		+	+	+	-	-	-
M		-	-	-	-	-	-
MAC		-	-	-	-	-	-
IN		+	+	-	-	-	-
Z		+	-	-	-	-	-
1		+	+	+	-	-	-
2		+	+	+	-	-	-
3		+	+	+	-	-	-
4		+	+	-	-	-	-
5		+	-	-	-	-	-
6		+	-	-	-	-	-
7		+	-	-	-	-	-
8		+	+	-	-	-	-
9		+	-	-	-	-	-
(cheese)		+	+	-	-	-	-
MAN							
sauerkraut		+	-	+	+	-	-
K							
human)		+	-	+	-	-	-
S 32							
astitis)		+	-	+	-	-	-
C 67							

XIII. Table of Characteristics of Strains Studied.

Strain.	Litmus milk. Reduction preceding coagulation.	Volatile acids. 0.1 N. acetic acid in 500 cc. skim milk culture.	Behavior on blood agar.	Final pH in glucose infusion broth.	Temperature-growth relations.		Survival of pasteurization.	Growth in methylene blue. Milk (0.02 per cent). 1:5000 Reduction and coagula- tion.	Virulence for white mice	Sucrose prefer- red to lactose.	Fermentation of carbohydrates.					
					Growth at 10° C.	Growth at 43° C.					Sali- cin.	Manni- tol.	Su- crose.	Raf- fin- ose.	In- ulin.	Gly- cer- ol.
X	Complete.	50. (1)	B-hemolysis.	4.2	+	+	+	+	Survival.	-	+	-	-	-	-	-
PD	"	"	"	4.3	+	+	+	+	"	(1)	+	+	+	+	+	-
G	"	11.50	Methemaglo- bin.	4.2	+	-	-	+	"	-	+	-	-	-	-	-
S	"	13.40	"	4.5	+	-	-	+	"	-	+	+	-	-	-	-
Sk	"	9.08	Indifferent.	4.2	+	+	+	+	"	-	+	+	-	-	-	-
E	"	12.60	Methemaglo- bin.	4.3	+	-	-	+	"	-	+	+	+	-	-	-
W	"	10.80	Indifferent.	4.3	+	-	-	+	"	-	+	+	+	-	-	-
M	"	(1)	Methemaglo- bin.	4.5	+	-	-	+	"	-	-	-	-	-	-	-
MAC	"	"	"	4.5	+	-	-	+	"	-	-	-	-	-	-	-
IN	"	"	"	4.2	+	±	-	+	"	-	+	+	-	-	-	-
Z	"	"	"	4.5	+	+	+	+	"	-	+	-	-	-	-	-
1	"	"	"	4.2	+	+	+	+	"	-	+	+	+	-	-	-
2	"	"	"	4.1	+	-	+	+	"	-	+	+	+	-	-	-
3	"	"	"	4.2	+	±	-	+	"	-	+	+	+	-	-	-
4	"	"	"	4.2	+	-	+	+	"	-	+	+	-	-	-	-
5	"	"	"	4.3	+	±	+	+	"	-	+	-	-	-	-	-
6	"	"	"	4.5	+	-	-	+	"	-	+	-	-	-	-	-
7	"	"	"	4.3	+	±	-	+	"	-	+	-	-	-	-	-
8	"	"	"	4.2	+	-	-	+	"	-	+	+	-	-	-	-
9	"	"	"	4.2	+	-	-	+	"	-	+	-	-	-	-	-
(cheese)	"	"	B-hemolysis.	4.1	+	+	+	+	"	(1)	+	+	-	-	-	-
MAN	Not reduced.	"	Indifferent.	4.2	+	+	-	-	(1)	+	+	-	+	+	-	-
(sauerkraut)																
K	"	"	B-hemolysis.	5.0	-	(1)	-	-	"	(1)	+	-	+	-	-	-
(human)	"	"	"	4.4	-	"	-	-	"	"	+	-	+	-	-	-
S 32	"	"	"													
(mastitis)	"	"	"													
C 67																

(1) Not determined.



## GENERAL DISCUSSION

All of the collection isolated from sour milk and from fermented dairy products would be included in the so-called Streptococcus lacticus group by the usual, casual treatment. It is shown in the above table, that most of these strains possess the following characteristics in common: reduce litmus in litmus milk before coagulation; coagulate milk; in glucose infusion broth reach final H-ion concentrations more acid than pH 5.0; grow at low temperatures; reduce methylene blue in milk containing 0.02 per cent of the dye; exhibit no pathogenicity to white mice; ferment carbohydrates in the following order of availability; dextrose, lactose, salicin, mannitol and sucrose; do not ferment glycerol. None of the strains fermenting sucrose prefer that substrate to lactose. All cultures tested showed the production of only small amounts of **volatile** acid in milk culture. Methemoglobin production was the usual behavior on the blood plate.

These results, together with those of Evans (1918) and of Sherman and Albus (1918), suggest that a large number of the streptococci concerned in the lactic acid fermentation of dairy products possess a certain number of characteristics in common. Whether such a collection represents a natural group is another question and it is not the purpose of this paper to propose any definite boundaries to the so-called Streptococcus lacticus group. The possible advantages to be accrued from at least a temporary recognition of certain types as a working basis for their further study have already been suggested.

On the other hand, the assumption and recognition of definite groups of streptococci entail **certain** disadvantages, depending in no small degree upon the characters chosen as **salient** boundary marks

of these groups. These disadvantages are evident in Jensen's recent classification of lactic acid producing streptococci, in which a large number of possibly closely related types are separated and defined. Likewise, it seems that Jones' suggestion of Streptococcus lacticus I and Streptococcus Lacticus II, not only should await an establishment of the Streptococcus lacticus group itself, but should be based on a more fundamental character, if indeed such a division is desirable at all. For these reasons, it must be admitted that work with a larger number of lactic strains than are reported here, might indeed show that the characters given above for the typical Streptococcus lacticus lead to like disadvantages in attempts to bound the larger lactic group of streptococci.

The value of any system of grouping streptococci is in a large measure dependent upon its usefulness as a working basis for further study of their economic application and of their sanitary significance. From such a standpoint, definite characterization of the lactic group might prove of very limited value to the medical bacteriologist. On the other hand, for the agricultural bacteriologist, more definite characterization would furnish a more intelligent basis of study and seems to be required. Frequently throughout the literature, certain physiological reactions are assigned to the Streptococcus lacticus group, with no attempt to establish any other characteristic of the strains involved, than that of acid coagulation of milk. It is in this connection that the various interpretations of the boundaries of the lactic group assume the greatest moment.

Even in the present incomplete knowledge of streptococcal relationships, the indiscriminate use of definite but meaningless

group names is not desirable. Streptococci actively coagulating milk may not be members of the so-called Streptococcus lacticus group, merely because they were isolated from dairy products. For meagerly described strains from milk, it would seem that more definite terminology than "milk streptococci" is to be questioned.

Two of the strains exhibited the Beta type of hemolysis on the blood plate and actively hemolyzed rabbit blood corpuscles in saline solution. The exhibition of hemolysis by these strains raises the interesting question of whether or not some of the hemolytic streptococci of milk would conform to Evans' more definite characterization of the Streptococcus lacticus group. In the absence of data on the relative amounts of lactic and volatile acid produced in the fermentation of milk, it is impossible to answer this question. It may be seen in the tabular summary, however, that the hemolytic strains agree with all of the other characters of the lactic group. It is possible that these strains, together with those reported by Davis (1918) and by Salter (1921), represent examples of the overlapping of present systems of nomenclature and classification of streptococci.

It is desired to call attention to the source of the two hemolytic strains described in this paper, and to their apparent fitness for the struggle for microbial supremacy in milk and milk products. The existence of such strains suggests that at times hemolytic streptococci may be the predominating type in some samples of milk even during the later periods of its handling. Again, such hemolytic streptococci may be added in large numbers as "starters" in the manufacture of various dairy products. These products, whether pasteurized before or after the inoculation of the "starter", would contain hemolytic streptococci in large numbers. While these strains are probably of no sanitary significance, the

temperature relations of such strains may explain cases in which large numbers of hemolytic streptococci are found in milk and milk products. In the usual method of grouping hemolytic streptococci these strains would be included in the "bovine" type and could be distinguished from hemolytic streptococci of human origin by the method of Avery and Cullen (1919).

## SUMMARY

A review of the literature on the lactic group of streptococci is presented, in which emphasis is placed upon the need for more definite information regarding the boundaries of this group.

The lactic strains studied were isolated from sour milk, "starters", and other fermented milk products, as probable sources of the so-called Streptococcus lacticus. These strains have been subjected to a number of tests which have been used by different authors in the description and differentiation of different types of streptococci. *tested at least twice, after*

From this study, we have reached the following conclusions:

1. The literature reports that many strains of the type usually dominant in sour milk, possess a number of common physiological characteristics. These may or may not represent a natural group. A summation of characteristics by a large number of workers may serve in the future recognition of the group as a type.
2. At the present time, there is no differential character which can be used as an independent test to distinguish this group. Certain characteristics seem to offer means of differentiating the lactic streptococci from certain other types, but different criteria must be used in different cases.
3. Two strains of nonpathogenic hemolytic streptococci exhibit characters which suggest that hemolytic strains may not only be present in milk or milk products, but may take an active part in the lactic fermentation of dairy products.



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Plate.

TYPES OF BEHAVIOR  
EXHIBITED ON BLOOD AGAR BY STREPTOCOCCI FROM SOUR MILK.

(Explanation of the Plate.)

- Fig. 1.  
Appearance of Blood Agar Colony of Indifferent Strain.

Strain SK. Colony after 48 hours incubation at 37° C.; showing no change of the blood corpuscles surrounding the colony.

- Fig. 2.  
Usual Appearance of Colony of Methemoglobin Producing Strain.

Strain S. Colony after 24 hours incubation at 37° C.; showing zone of discolored corpuscles surrounding the colony.

- Fig. 3.  
Appearance of Colony of Methemoglobin Producing Strain After Refrigeration.

Strain S. Colony after 48 hours refrigeration at 10° C., following 48 hours incubation at 37° C.; showing a clear zone surrounding an inner ring of non-hemolyzed but discolored corpuscles next to the colony. The clear zone appears upon refrigeration of blood plates of methemoglobin producing strains after previous incubation at 37° C. This phenomenon is termed the alpha type of hemolysis by Smith and Brown.

- Fig. 4.  
Appearance of Blood Agar Colony of Beta-Hemolytic Strain from Sour Milk.

Strain X. Colony after 18 hours incubation at 37° C.; showing a wide clear zone as the result of hemolysis of the blood corpuscles surrounding the colony. This is termed the Beta type of hemolysis by Smith and Brown.

- Fig. 5.  
Appearance of Blood Agar Colony of Beta-Hemolytic Human Strain.

Strain S32. Hemolytic human strain included for comparison with the hemolytic sour milk strain shown in Fig. 4.

PLATE

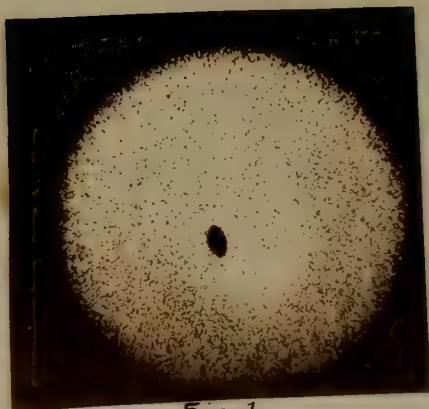


Fig. 1.

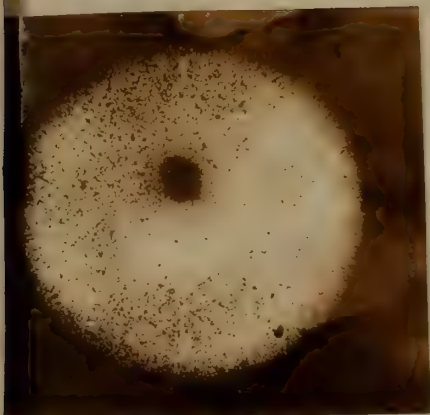


Fig. 2.

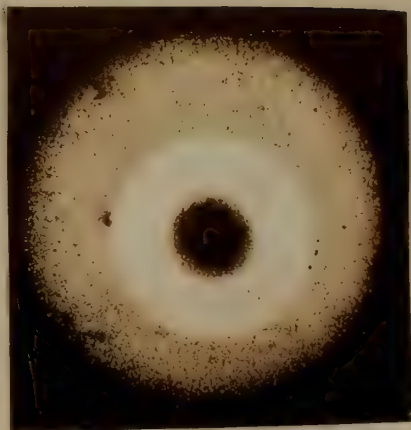


Fig. 3.

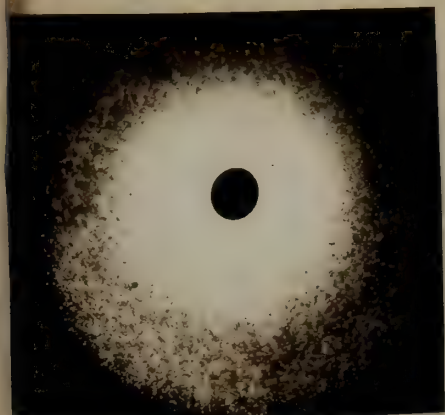


Fig. 4.



Fig. 5.



PART III.

"A COMPARATIVE STUDY OF THE SEPTOLYTIC  
ACTIVITY OF DIFFERENT TYPES OF STREPTOCOCCI.  
WITH SPECIAL REFERENCE TO THE INFLUENCE OF  
ENVIRONMENTAL CONDITIONS".

### Part III.

"A COMPARATIVE STUDY OF THE PEPTOLYTIC ACTIVITY OF DIFFERENT TYPES OF STREPTOCOCCI, WITH SPECIAL REFERENCE TO THE INFLUENCE OF ENVIRONMENTAL CONDITIONS."

#### INTRODUCTION.

- I. Activities of Streptococci in Nature.
- II. Significance of Proteolytic Action of Streptococci.
- III. Grouping of Streptococci as a Basis of Physiological Study.
- IV. Groups Chosen for Study.

#### INVESTIGATION.

##### Purposes.

A comparison of the peptolytic activity of different types of streptococci.

A recognition of the significant influence of environmental conditions upon the biochemical activity of microorganisms results in the division of the purposes of the study into the following two sections.

- (A.) A comparison of the relative influence of environmental conditions upon the peptolytic activity and other life processes of different types of streptococci.

Here, the study is focused upon the establishment of optimal environmental conditions for the peptolytic action of the different streptococci.

- (B) A comparison of the peptolytic activity of different types of streptococci under the foregoing standardized optimal environmental conditions.

## General Methods.

### I. Chemical Methods.

1. Determination of amino nitrogen.
2. Determination of ammonia nitrogen.
3. Control of volume of tests.

### II. Bacteriological Methods.

1. Condition of inocula and purity of tests.
2. Methods used for the approximate comparison of the relative numbers of active cells, or for comparison of the "general growth condition" or vitality of different cultures of the same strain of streptococcus.

## Section A.

THE RELATIVE INFLUENCE OF ENVIRONMENTAL CONDITIONS UPON THE LIFE PROCESSES OF DIFFERENT TYPES OF STREPTOCOCCI FOR THE PURPOSE OF STUDYING THEIR PEPTOLYTIC ACTIVITY.

### I. Influence of H-ion Concentration upon the Peptolytic Action and Other Life Processes of Different Types of Streptococci.

1. Influence of H-ion concentration upon amino nitrogen increases.
2. Influence of H-ion concentration upon the growth and viability of different types of streptococci.
  - a. Relative acid tolerance of the different types.
  - b. Influence of H-ion concentration upon the rate of growth and longevity of different types of streptococci.
3. Summary and discussion of the influence of H-ion concentration upon the life processes of the different type strains.

## II. Influence of the Stage of Growth of the Culture Upon the Increases in Amino and Ammonia Nitrogen.

### 1. Experimental.

### 2. General discussion of the relation of growth stage of the culture to increases in amino and ammonia nitrogen.

- a. Nature of nitrogen distribution in peptone broths.
- b. Meaning of amino and ammonia nitrogen increases in bacterial cultures.
- c. Possible sources and methods of formation of amino and ammonia compounds in peptone broth cultures of streptococci.

## III. Influence of Temperature Upon the Peptolytic Action and Upon Other Life Processes of Different Types of Streptococci.

1. Influence of different temperatures upon amino nitrogen increases.
2. Comparison of amino nitrogen increases at 37° and 41° C.
3. Influence of size of inoculum upon amino nitrogen increases at optimum temperature, and at temperatures above the optimum.
4. Influence of temperatures above the optimum upon the final H-ion concentration in glucose broth.
5. Influence of temperature upon the rate of growth, and upon the activity and vitality of different types of streptococci.
  - a. Relative rate of growth of the different streptococci at different temperatures.
  - b. Comparison of the relative activity and vitality of cultures of the different types of streptococci when incubated at different temperatures.
6. Summary and discussion of the influence of temperature upon the life processes of the different types of streptococci.

IV. Influence of Oxygen Concentration Upon Amino Nitrogen Increases.

V. Optimal Environmental Conditions for the Different Types as Shown by the foregoing Study.

## Section B.

COMPARISON OF THE PEPTOLYTIC ACTIVITY OF DIFFERENT TYPES OF STREPTOCOCCI UNDER THE VARIOUS STANDARDS OF OPTIMAL ENVIRONMENTAL CONDITIONS.

I. Preliminary Statements.

1. Relation to preceding studies.
2. Description of groups and strains studied.

II. Comparison of Amino and of Ammonia Nitrogen Increases Exhibited by Different Members of the Lactic Group, and by Strains of Other Types of Streptococci.

III. Comparison of Amino Nitrogen Increases Effected by Different Types of Streptococci.

IV. General Discussion of the Comparative Peptolytic Activity of Different Types of Streptococci.



GENERAL SUMMARY.

ACKNOWLEDGMENTS.

BIBLIOGRAPHY.

## INTRODUCTION.

### I. Activities of Streptococci in Nature.

Streptococci appear in the most divergent rôles in nature. The lactic group of streptococci are recognized as important agents of lactic acid fermentation. As such, they are employed in the production of butter, certain cheeses, various fermented milks and other dairy products. Again, the activities of certain streptococci are involved in the preparation of many fermented plant food stuffs, as silage, sauerkraut and certain pickles. Some streptococci are frequently associated with severe human pathological conditions. Others are commonly found in the udders of cows and are associated with mastitis; still others appear to be harmless inhabitants of the alimentary and respiratory tracts of man and other animals. Due to the obvious importance of the various types of streptococci, more definite knowledge concerning their metabolism should be obtained.

Knowledge of the physiological properties of the organism is essential to an appreciation of the actual means by which different streptococci produce important changes, whether in milk, in butter, in cheese, or in the human body. It is only by the gradual illumination afforded by cumulative investigations that their fundamental life processes, and the conditions controlling their operations can be interpreted. Such an interpretation must be the foundation of intelligent application of their activities in agriculture, and, likewise, must underlie the

intelligent control of the insidious activities of other kinds of streptococci in the production of disease.

## II. Significance of Proteolytic Action of Streptococci.

Among the varied processes involved in microbial activities, those concerned in the nitrogen metabolism of streptococci have received comparatively little attention. Altho', under certain conditions, streptococci are essentially acid forming microorganisms, their proteolytic activities assume importance from many aspects. Following from the importance of proteolytic changes in systems in which streptococci may be the active agents, the significance of this phase of their metabolism ramifies from a more or less common center into processes of moment in agriculture, medicine, and public health.

## III. Grouping of Streptococci as a Basis of Physiological Study.

Certain important considerations must precede the active investigation of any of the physiological processes of different members of the streptococcus genus. Before assigning definite functions to certain streptococci, it must be more or less definitely understood to what particular members of the genus, the results of such researches may be projected.

Notwithstanding the probable fallacies of a systematic classification of streptococci, an intelligent pursuit and interpretation of investigations of any of their physiological processes require a certain differentiation between different

streptococci. In spite of the fundamental differences which would seem to exist among microorganisms which function in such different ways as do many of the streptococci, this genus has been unique in its resistance to the usual differential methods of the bacteriologist. For this reason, methods of differentiation of streptococci are usually limited to distinguishing between certain large groups. These groups and their contents have varied with the method of classification and with the purpose of the investigator.

#### IV. Groups Chosen for Study.

That there are many of these groups must follow from the varied activities of streptococci. Those chosen for study in this investigation represent only four of the most important divisions : (1) the so-called "lactic" group of sour milk, (2) the "human" hemolytic group, (3) the "bovine" hemolytic group, (4) the "cheese" hemolytic group.

The "lactic" type represents the large group of streptococci which function as agents of the lactic fermentation of milk and milk products. The "human" type of hemolytic streptococci represents a large group usually from human sources and frequently associated with pathological conditions. This group is distinguished from other hemolytic streptococci by the lower H-ion concentration reached in glucose broth cultures (Avery and Cullen). The "bovine" type or "high acid" group of hemolytic streptococci probably includes strains from various sources in nature. In this investigation, the "bovine" group of hemolytic streptococci is represented by strains obtained from the udder of cows. Such

strains are frequently associated with mastitis. The "cheese" hemolytic type represents a large group of hemolytic streptococci which seem to be quite commonly present in cheese.

These groups have been established by bacteriologists as convenient bases for further study. They have been proposed by the original authors as a convenient means of treating in a collective manner, certain large collections of strains having a number of common characters. The fact that their members also have a more or less common economic importance lends value to these divisions and furnishes a logical basis for collective treatment.

Comparative studies of these different types of streptococci seem particularly desirable in the face of their obvious economic importance. The importance of the lactic group has been developed in Parts I and II of this thesis. The human type of streptococci is perhaps most important in medicine. However, its close relationship to the lactic streptococci renders comparative studies of the two types an essential step in the gradual development of a future true understanding of the rôle of streptococci in agriculture. Much the same may be said in the case of the bovine or mastitis group of streptococci. The possible importance of the large number of hemolytic strains included in R. C. Avery's cheese group, can not be overlooked in a survey of the probable biological agents of cheese ripening. These four groups are particularly deserving of comparative studies because of the fact that not infrequently all four of these



types of streptococci may be found in one sample of market milk. Here, a knowledge of the conditions controlling the successful operation of their life processes, will furnish some idea of the possibilities of their continued growth in the milk or in products manufactured from milk, - in short, their significance in dairy foods.

## INVESTIGATION.

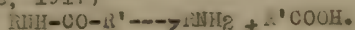
## Purposes:

The general purpose of the investigation is a study of the peptolytic activity\* of different types of streptococci.

While our principal interest is centered upon the lactic streptococci of dairy lactic fermentations, the peptolytic action of the lactic streptococci is compared with other important types of streptococci which are closely related to the lactics, and which also are of undoubted significance in dairy products.

A comparison of the extent or "activity" of any biochemical process requires a strict definition of the system. This is true in the case of the study of any group of microorganisms; it is especially required in the case of streptococci, which are among all microorganisms perhaps the most responsive to environmental conditions. Hence, a comparative study of the peptolytic activity of different types of streptococci requires a recognition of the important influence which certain factors in the environment may exert upon the action of the peptolytic processes of microorganisms.

\*The term "peptolytic activity" is used in this paper as an expression of the increases in amino nitrogen which follow the bacterial cleavage of the various compounds present in "peptone". The nitrogen of the mixtures of partially hydrolyzed protein products known commercially as "peptone", is largely in the form of peptide nitrogen. By peptide nitrogen is meant nitrogen found in the peptide linkings, the  $-CO-NH-$  groups that link the different amino acids together in peptides, proteins or intermediate products. The cleavage of the compounds included in "peptone" may thus be termed "peptolysis", - a term indicative of the nature of the substances undergoing hydrolysis. The process of "peptolysis" consists in the splitting of the peptide groups, from each of which is generated a carboxyl group and an amino group, as indicated in the following equation (Van Slyke, 1917)



For further discussion of the relations involved see Van Slyke (1917), and pp. of this paper.

14.  
With these facts in mind, the purposes of the investigation naturally fall under the following two direct objects -- of sequent significance rather than of coordinate value.

(A) A comparison of the relative influence of environmental conditions upon the peptolytic activity and other life processes of different types of streptococci.

In this section, the influence of environmental conditions is investigated for the purpose of establishing the optimum conditions for the peptolytic action of the different streptococci.

In addition to the establishment of the optimum conditions as an intelligent basis upon which to define the systems in which the comparisons are to be made in Section B, Section A will also present data of independent value.

(B) A comparison of the peptolytic activity of different types of streptococci under the foregoing standardized optimum environmental conditions.

In this section, the peptolytic action of different streptococci is compared under the optimal environmental conditions which were established in Section A.

## General Methods.

### I. Chemical Methods.

Measurements of amino nitrogen and of ammonia nitrogen were chosen as the basis of the study of the peptolytic action of the streptococci. The study was limited to the production of these products in meat infusion "peptone" broth, the usual medium of cultivation of these organisms. While the results so obtained may not apply to the action upon intact proteins themselves, or even upon more complex protein derivatives, they at least serve as an index of the action of streptococci upon peptides and other constituents of commercial "peptone".

#### 1. Determinations of amino nitrogen.

Amino nitrogen determinations were made by the nitrous acid method of Van Slyke. The micro apparatus described in 1913 was used with the 3 cc. gas burette described in 1915. Direct determinations were made from 2 cc. samples of the culture.

Conditions and precautions prescribed by the author of the method were observed. In addition, all determinations were made under temperature conditions varying only between 22° and 25° C. Duplicate determinations were made of each test.

Amino nitrogen is expressed in tables given in the text as mg.  $\text{NH}_2\text{-N}$  per 100 cc.

#### 2. Determinations of ammonia nitrogen.

Ammonia was determined by the method described by Van Slyke and Cullen (1914, 1916). Careful attention was given to the conditions governing the accuracy of the method (Van Slyke and Cullen, 1916).

10 cc. of saturated solution of  $K_2CO_3$  were added to 10 cc. of the sample under analysis. Two drops octyl alcohol were added to prevent foaming and ammonia was aerated into 25 cc. of .02 N HCL. The acid neutralized was determined by titration with 0.02 N NaOH, using sodium alizarin sulphate as indicator. Ammonia nitrogen is reported in tables in the text as mg.  $NH_3$ -N per 100 cc.

### 3. Control of volume of tests.

All cultures were incubated in moisture saturated incubators; vaporation of tests was controlled by weight.

## II. Bacteriological Methods.

### 1. Condition of inocula, and purity of tests.

Unless stated otherwise, all test cultures received inocula of 0.1 cc. of 12- to 18-hour broth cultures which had been "invigorated" by at least 4 successive 12-hour transfers.

The purity of all test cultures was controlled by microscopic examinations and cultural tests. The latter consisted of subcultures upon infusion agar plates for detection of contaminations, or of milk cultures to distinguish between the type strains themselves.

### 2. Methods used for the approximate comparison of the relative numbers of active cells, or for comparison of the "general growth condition" or vitality of different cultures of the same strain of streptococcus.

#### a. The plate method.

In the first of our experiments, the number of viable cells is approximated by the usual plate method. This is the commonly accepted method in general microbiological studies. At best it can present but approximate figures. The errors inherent in the method are particularly evident in the case of long chained



streptococci. Possibly in part due to this fact, the plate method did not prove very satisfactory in the case of tests of the "human" and "bovine" strains. (This difficulty is also reported by Foster (1921)). Moreover, considerable labor is involved in making a determination of a large series by this method, as plates should be poured at least in triplicate to insure any degree of accuracy at all. In case of tests in which the worker has no previous knowledge of the approximate number of cells present in a sample, a still larger number of plates at different dilutions is necessary.

For these reasons, the following method was introduced in the latter part of our work.

b. Principle of method used in part of this investigation.

Streptococci are essentially acid forming organisms. When these organisms are present in systems presenting an easily fermented sugar, they utilize that substrate as the primary source of energy. Upon the introduction of these organisms into glucose broth, their reproduction is dependent upon the energy yielded by the lactic fermentation of the sugar. This reaction yields a product, increases of which are easily detected. Hence, these relations may be regarded as established: (1) the fermentation of glucose is the basic and essential life process of lactic acid producing streptococci; (2) this reaction yields a product whose accumulation can be measured accurately.

It remains to establish the conditions determining the rate of acid production. These relations have been discussed in detail in Part I of this paper ("Theoretical Progress of Lactic Acid Fermentation".) The following is but a brief statement of the

general principles determining the rate of production of the acid product. The acid produced in glucose media by growth of streptococci may be regarded as dependent upon the multiplication of cells. The multiplication, of course, is dependent upon the number of cells in the initial inoculum, and will follow in a general way the curve of organic growth, during the earlier period of the fermentation. In this earlier period before inhibiting factors influence the curve of growth, the rate of acid production in a definite and ideal system brought about by a defined fermenting agent is then dependent upon the number of active cells present in the initial inoculum which is introduced into the fermentation system. These relations will hold for a particular strain of streptococci, provided the organisms introduced into the test system are able to begin multiplication at an equal rate.

The conditions influencing the initial phenomena of growth are discussed in the reference given above. However, it may be briefly stated here that, at least in an ideal culture medium, growth will be initiated at an orderly rate, provided the inocula are taken from young cultures which have not been subjected to unfavorable environments.

The principles of this method developed above may be restated as follows: (1) the fermentation of glucose in glucose media is the fundamental, energy obtaining life process involved in the growth of lactic acid producing streptococci; (2) this reaction yields a product whose concentration can be measured accurately; (3) the rate of production of this reaction product is a function of the rate of multiplication of the bacteria introduced, provided tests are limited to the early stages of the reaction in the ideal fermentation systems described above.

### c. Manipulation of the method.

It is desired to compare either the relative number of active cells, or the "general growth condition" of different cultures of the same strain of streptococcus. A series of cultures of the same strain are to be tested with either of the above objects in mind.

Equal amounts of each culture in the series is introduced into equal amounts of glucose broth. The original inocula represent equal volumes of each test in the series. The glucose broth represents an ideal fermentation system in which the rate of acid production is dependent upon the multiplication of the bacteria introduced. The increase in the concentration of the acid is detected by an indicator present in the system. The time required for the production of acid sufficient to give the indicator a definite color is recorded.

The detailed technique follows: Each culture in the test series is shaken thoroughly to ensure the removal of a representative sample. One cc. of each member of the test series is introduced into 100 cc. of sterile physiological salt solution. These dilutions are shaken thoroughly. (Dilutions of test cultures are employed merely as a more convenient and probably more accurate means of obtaining a small sample of the original test.) One cc. of the dilution is then introduced into duplicate tubes of 12 cc. of glucose infusion broth containing 1.0 per cent Andrade indicator. (The glucose broth is tubed in measured quantities; test tubes of uniform bore are used.) The inoculated glucose broth is shaken thoroughly to distribute the inoculum. These glucose broth test cultures are then incubated at  $37^{\circ}$  C. in a Wassermann bath. (The glucose broth used in the test is brought to a temperature of  $37^{\circ}$  before inoculation and is maintained at that temperature throughout the manipulation.)

Observations are made at 15 minute intervals. The glucose broth tests are shaken several times during the incubation period. The time required for the attainment of a distinct pink color of the Andrade indicator is reported in the case of each of the tests. The color of the tests was compared with a strip of pink paper as a means of obtaining an end point of approximately equal value. The advantages and disadvantages of this indicator will be discussed in following paragraphs under the advantages and disadvantages of the method itself.

#### d. Use of the method.

The uses of any method of comparison are dependent upon its principles. The principles of this method have been stated above. For the convenience of the reader and the interpretation of the comparisons in which it has been employed, they may be restated as follows: Under equal temperature conditions, the time required for the production of a definite concentration of the reaction product is dependent upon the speed of the reaction. The speed of the reaction of lactic fermentation in its earliest period is dependent upon the number of cells and the multiplication of the fermenting agent. The method as a whole then is dependent upon the conditions influencing multiplication. Its uses then are dependent upon these conditions.

The conditions determining multiplication have been given in detail in Part I of this paper. ("Theoretical Progress of Lactic Acid Fermentation") Nothing more will be given here than a statement of the conditions under which the method has been used.

I. Approximate comparison of the numbers of active cells in different cultures of the same streptococcus.

This case requires the strictest definition. Not only must all of the conditions involved in the preceding discussion be observed, but comparisons must also be limited to certain definite series of cultures.

Cell multiplication, and consequently acid production, are dependent upon the ability of the number of cells inoculated to initiate growth. In ideal environments, "lag" and differences in rate of multiplication are dependent upon the vigor of the cells. Hence, the acid production and multiplication of the bacteria will follow the curve of growth only if the cultures are seeded with young cells which have not been subjected to previous unfavorable conditions.

Hence, for reasons given above, the method described can be used for a comparison of numbers of cells in the case of young cultures. Under the conditions prescribed it is believed that



the time required for the production of a definite amount of acid, is a function of the initial number of cells.

This set of conditions was maintained in the study of the influence of different temperatures upon the rate of growth. Here, a series of flasks containing equal volumes of broth were held at different temperatures. Each flask then received equal inocula of the lactic streptococcus. After 12 hours incubation, the relative rate of growth in the different members of the series was compared. Figures were obtained which show the same general relations as those obtained by plate counts made at the same time upon the same series.

Cultures of greater age than 12 hours probably should not be tested. It is also unknown whether this method would distinguish smaller differences in the rate of growth in series whose members differed by small increments. With the wide zones tested, the method gave satisfactory results.

## II. Comparison of "general growth condition" of different cultures of the same streptococcus.

This set of conditions does not require as strict definition as the former. "Lag" and similar phenomena involved in bacterial growth and acid production do not limit the value of the method in this instance. In fact, they are of actual service in the application of the method for the comparison of the "general growth condition" of a series of cultures of the same fermenting agent.

The "general growth condition" is assumed to represent not only the number of viable cells but also their relative vitality and activity. The number of cells and also their condition, is dependent upon the environment and upon the period to which they have been exposed to this environment. Both of these relations are involved in the speed of multiplication and of acid production of inocula taken from a series of different environments.

Hence, under this set of conditions, the method can be applied in a comparison of the relative influence of different environments for a given period of time. It may also be used in the comparison of the influence of exposure to a given environment for different periods of time.

Both of these relations are evident in the experiment in which the second set of conditions has been maintained, in a study of the relative influence of different temperatures upon the growth and physiologic activity of different types of streptococci. This method should furnish a ready means of comparing the "general growth condition" or vitality of different cultures of the same strain. Both rate of acid production and multiplication are dependent on the condition of the inoculum (age, size, and resistance to previous environment), which is in fact the definition of the "general growth condition" or vitality.



It is probable that this method furnishes a more direct evaluation of differences in bacterial vitality than can be furnished by the plate method. The later method merely presents an approximation of the relative number of viable cells present which are ultimately able to produce a colony of appreciable size, while the above described method presents an approximation of the relative condition of the cells.

e. Disadvantages of the method.

The method at best is crude. It can serve only in the comparison of the same strain, as differences exist in the fermenting capacity of different strains. The introduction of large inocula into the glucose Andrade broth must be avoided, in cases where significant changes in the reaction of the broth would be effected in that way. The choice of Andrade indicator is open to serious questions. The change in color of this indicator is not instantaneous by any means. Exact determinations of H-ion concentration cannot be made. (It is probable that more accurate data could be obtained by the use of brom cresol purple and a definitely standardized colorimetric comparison.)

f. Advantages of the method.

The chief advantage of the method is its convenience. Less time, labor and materials are required than for carefully controlled plate counts.

Disadvantages following the choice of Andrade indicator have been mentioned. However, it has the following advantages. The presence of this indicator in the glucose broth apparently exerts

no harmful influence upon the growth of streptococci, as this indicator is commonly used in tests of carbohydrate fermentation. The fact that Andrade indicator changes in color at H-ion concentrations not far removed from the neutral point is also a point in its favor. (Fennel and Fisher reported definite magenta color is exhibited by Andrade indicator at a ph of 6.6 to 6.8.)

There are two reasons for the use of an indicator whose color change is not far removed from the neutral point. (1) At high H-ion concentrations the products of growth are imposing their influence upon the curves of growth and of acid production. Hence, acid production is a function of the initial number of cells only during the early phase of the fermentation. (2) At later periods in the course of fermentation, the differences in concentration of acid produced by inocula of different sizes are constantly decreasing. The greatest differences are manifest in earlier periods of the fermentation. (This relation is evident in the results reported in the work of Foster (p. 182, fig. 3.), which appeared several months after the completion of this investigation. The relations involved here are also discussed in detail under "Theoretical Progress of Lactic Acid Fermentation" in part I of this paper. Curves given there also furnish further support to the above statements.)

The method is not presented as a method, but merely as the means by which results reported in this study were obtained. In the following text, this method is termed the "Andrade Test".

## INVESTIGATION.

## Section A.

THE RELATIVE INFLUENCE OF ENVIRONMENTAL CONDITIONS UPON THE LIFE PROCESSES OF DIFFERENT TYPES OF STREPTOCOCCI, FOR THE PURPOSE OF STUDYING THEIR PEPTOLYTIC ACTIVITY.

I. Influence of H-ion Concentration Upon the Peptolytic Action and Other Life Processes of Different Types of Streptococci.

The influences of H-ion concentration upon the functioning of micro-organisms have proven fruitful subjects of study. These investigations have shown that the true acidity of the environment conditions, and, in many cases, determines, the extent and direction of the various processes involved in microbial metabolism.

In a study of the proteolytic activity of different types of streptococci, the influence of this factor assumes particular moment. The sphere of action of their proteolytic enzymes is limited to certain zones of H-ion concentration; again, even within the zone permitting their action, the degree of activity is conditioned by smaller increments of change in the true acidity of the system. The proteolytic enzymes can not function until they are first elaborated and their elaboration is dependent upon the successful and luxuriant growth of the cells. Consequently, the influence which H-ion concentration exerts upon all of the life processes of streptococci is reflected in a less direct but still pertinent manner, upon the proteolytic changes induced by their enzymes. All of these relations project themselves into many

processes in the different fields in which streptococci are important.

### 1. Influence of H-ion concentration upon amino nitrogen increases.

The influence of the H-ion concentration upon the increases in amino nitrogen effected by the different types of streptococci, was studied by comparing the changes brought about by the type strains in systems of different pH value. Comparatively wide zones were chosen as a means of determining variations in the general behavior of streptococci in different H-ion concentrations. Changes in the initial reaction of even highly buffered media occur in plain broth cultures of streptococci, which would render very difficult the comparison of the influence of initial differences of smaller increments of pH. (Itano, 1916).

#### Procedure: (Experiment 1.)

Preparation of medium: Infusion broth prepared as follows was used as the medium in this experiment:

For each desired liter of medium, 500 g. chopped lean meat was added to 1,000 cc. of distilled water. After the mixture had been infused 12 hours in the ice-box, it was strained thru cheese cloth. The infusion was heated one half hour at 100° C. and then filtered. The volume was corrected and the infusion autoclaved in flasks. One per cent of Difco peptone and 0.5 per cent NaCl was added to the meat infusion. After ingredients were in solution, the broth was autoclaved for 5 minutes at 15 lb. pressure and then filtered. The filtered broth was then sterilized in 600 cc. quantities, at 116° C.

The broth was adjusted to the desired pH zones as follows: Sample flask of the unadjusted broth was titrated colorimetrically, to the desired pH values by use of the standard solutions and indicators described by Clark and Lubs (1917) for the respective pH ranges.

The calculated amount of 1.0 N HCl or 1.0 N NaOH was added to the respective flasks of broth. Amounts added are given in Table I. Flasks were then shaken and incubated at 37° C. for



several hours.

The broth was then distributed into flasks in 100 cc portions, with aseptic precautions. These test flasks were incubated at 37° C. to insure sterility and to stabilize the medium. After 48 hours incubation, the H-ion concentration of the medium was determined electrometrically thru the kindness of Dr. A. Itano.

Table I.

Preparation of Broth of a Series of pH Zones.

Infusion broth, unadjusted, was autoclaved. Following amounts of alkali and acid were added with aseptic precautions. pH determined after 2 days incubation at 37° C. pH values represent the H-ion concentration at time of inoculation.

Desired pH Zone	Amount added per 100 cc. of unadjusted broth.		Actual pH.
	1.0 N HCl	1.0 N NaOH	
4.5	1.40	----	4.45
5.5	0.50	----	5.40
6.5	----	1.05	6.70
7.5	----	1.70	7.55
8.5	----	3.12	8.63

(Experiment 1.)

One flask of medium at each pH value was inoculated with 0.1 cc. of an 18-hour broth culture of the representative strain. The cultures were then incubated at 37° C. Samples were removed at the intervals stated and determinations made of the amino nitrogen.

The results are given in Table II.

The significance of these results will be discussed in the general review of the experiments upon the influence of H-ion concentration upon the life processes of different types of streptococci.



Table II.

## Influence of H-Ion Concentration upon Amino Nitrogen Increases.

Broth of different pH value received inocula of 0.1 cc. of an 18-hour broth culture of each type strain. Results of  $\text{NH}_2\text{-N}$  determinations are expressed below in mg./100 cc.

	pH Zone.*	Total $\text{NH}_2\text{-N}$ .			Increase in $\text{NH}_2\text{-N}$ .
		24 hr.	168 hr.	Control.	
Lactic	5.5	54.3	55.7	53.5	2.2
	6.5	54.0	56.5	52.2	4.3
	7.5	52.7	55.6	51.9	3.7
	8.5	51.1	51.2	51.2	---
Human	5.5	53.3	53.6	53.5	---
	6.5	53.0	59.5	52.2	7.3
	7.5	52.6	60.8	51.9	8.9
	8.5	52.0	51.8	51.2	0.6
Bovine	5.5	53.0	55.5	53.5	2.0
	6.5	53.9	55.5	52.2	3.3
	7.5	53.3	55.9	51.9	4.0
	8.5	51.3	51.5	51.2	0.3
Cheese	5.5	54.8	56.8	53.5	3.3
	6.5	57.0	61.7	52.2	9.5
	7.5	56.5	60.2	51.9	8.3
	8.5	51.1	51.1	51.2	0.9

\*Actual initial pH values of the series are given in Table I.

## 2. Influence of H-ion concentration upon the growth and viability of different types of streptococci.

In such tests as those just reported, the influence of different initial pH zones upon "proteolytic" action is to a large extent a reflection of the effect of initial reaction upon growth. The following experiments were conducted to show in a more specific manner, the influence of different initial H-ion concentrations upon the growth and vitality of the different streptococci.

### Procedure:

#### (Experiment 2). Acid Tolerance.

In the preceding experiment, the cultures at pH 4.5 had shown no growth either by turbidity or by increases in  $\text{NH}_2\text{-N}$ . In this experiment the test of the influence of this reaction zone was limited to test of the vitality of the different types after varying periods of exposure to this zone of H-ion concentration. Plates were poured at stated intervals, to give some idea of the approximate rate of death of the different types. No attempt was made to follow the course of the killing reaction. This series received inocula of 1.0 cc. of 18-hour broth cultures.

Plate counts were made of the cultures from which these inocula were taken. The data obtained indicated that the following number of cells represents the initial concentration of bacteria per cc. of the test broth. \*

Lactic	400,000
Human	60,000
Bovine	120,000
Cheese	600,000

\*The variation in the number of cells represented in the above figures is more apparent than real. It is probable that the actual initial concentration of the cells of the different streptococci is much more uniform than would appear from the above figures. These differences in numbers as determined by plate counts are due to recognized and inherent errors in counts made by the plate method. The divergence in numbers is exactly what would be expected from the difference in length of chains represented by the different type strains. The much lower values always obtained in plate counts of long-chained streptococci (such as the above human and bovine strains) is a common laboratory observation. As the number of cells is compared only with numbers of cells of the same strain, this apparent discrepancy in the initial concentration of the different streptococci does not vitiate to any extent the value of the results given in Tables IV and V.

As a more ready means of comparison of the resistance of the different types to high H-ion concentrations, the counts made in the tests have all been reduced to a common basis of an equal initial concentration of 100,000 cells per cc. The results presented in Table IV are expressed upon this basis.

(Experiment 3.) Rate of growth and longevity.

In the other pH zones, plates were poured at the intervals shown in Table V, in an attempt to present comparative figures representing the relative rate of growth of the different types when introduced into systems of different pH values. The tests made at the later periods furnish some idea of the relative longevity of these types. Cultures in this series were inoculated with 0.1 cc. of 18-hour broth cultures.

Results are given in Table V.

Plate counts were made of the cultures from which inocula were taken, at the time of inoculation of this series. Numbers so obtained were divided by the number of cc. of the test culture. The result is expressed in the table as "Probable initial number of cells present".\*

Media used:

Media used for the tests: Broth of a series of pH values were prepared as described in the preceding experiment, except that the colorimetric method was used in determining the H-ion concentration. This broth was distributed into flasks in 50 cc. portions.

Media used in determining the number of cells: 1.0 per cent glucose infusion agar, pH 7.4, was used in making the counts. (This medium supported the growth of all strains.) In the pH 4.5 series, tests for vitality were also made by introducing 0.1 and 1.0 cc. of the inoculated broth into 10 and 50 cc. of glucose infusion broth, pH 7.6, containing 0.2 per cent sodium phosphate.

Table IV.

Relative Acid Tolerance of Different Types of Streptococci.

Comparison of the viability of different streptococci in pH 4.5 broth. Number of cells viable per 100,000 of initial concentration, after the stated intervals of exposure to pH 4.5 at 37° C.

Exposure. (hrs.)	Human.	Bovine.	Lactic.	Cheese.
5	100	9,000	48,000	90,000
10-12	---	1,200	10,000	120,000
24	---	-----	800	66,000
72	---	-----	-----	80,000
120	---	-----	-----	30,000

Final H-ion concentration  
in glucose broth cultures of  
the same strains.

pH	5.0	4.4	4.2	4.1
----	-----	-----	-----	-----

\*The statements made in footnote on preceding page will explain the apparent divergences in number of cells used as inocula.

a. Relative acid tolerance of the different types:

The results given in Table IV show the relative resistance to high H-ion concentrations exhibited by the different types of streptococci. The order of resistance of the different types to high acidities is as follows: cheese, lactic, bovine and human.\* The actual rate of death, of course would be less at lower temperatures than at 37° C., but it is doubtful if this would displace the above relations between the different types.

As the pH value of the medium used in this experiment closely approaches the acidity of sour milk, the above order of acid resistance of the different streptococci possesses obvious significance. In such a medium, one would expect the human streptococcus to die rapidly. The lactic and cheese strains would persist longer than the bovine or mastitis types. The cheese strain is much more resistant than the lactics and would not decrease in numbers so rapidly in sour milk, acid cheeses and similar acid systems.

\*While it is true that "plate counts" seem to show a great divergence in initial concentration of cells, it must be remembered that these large differences are due to the plate method itself, which enumerates slumps and groups as single organisms. It happens that these differences are also in the same order as the relative acid tolerance. One must admit the possibility of influence of the size of the inoculum and the initial concentration of the cells upon the rate of death of cells, but it is obvious that slight differences in initial concentration can not produce such wide divergences in time required for the end-point of the killing reaction, as are represented in Table IV.



These general relations to high acidity are more or less in order with the final H-ion concentrations reached in glucose broth cultures of the different types. From the final pH attained by the human strain, its rapid rate of death in broth of higher H-ion concentrations is to be expected. However, the specific effect of the H-ion is not so readily interpreted as to permit the assumption of parallel relations between the final "fermentation limit", and the tolerance of the organism to high acidities when introduced as inocula from cultures which have been growing in the optimum pH zone. In experiments with the pneumococcus, Avery and Cullen (1919 b) have demonstrated this in a conclusive manner. (These relations are discussed in detail in Part I of this paper, under "Influence of H-ion Concentration upon the Lactic Acid Bacteria", and "End Point of Lactic Acid Fermentation".) This is evident in a comparison of the final H-ion concentrations of the bovine, cheese, and lactic strains.

That no parallel relation exists between the final "fermentation limit" in glucose broth and the absolute tolerance of the organism to high acidities, is especially obvious in the case of the cheese and lactic strains. The cheese strain has a "fermentation limit" of pH 4.1 as compared to pH 4.2 exhibited by the lactic strain. In spite of the slight difference in this character, there is a striking difference in the actual resistance of these two strains in broth of pH 4.5 value. (It is shown in Table IV that the cheese strain was not greatly reduced in numbers in the pH 4.5 broth at the end of the time required for complete disinfection of the lactic, under the conditions of this experiment.)



The general relations in the acid tolerance of these types of streptococci are all that may be interpreted from the above experiment- and these, to a certain extent, only for the conditions obtaining in this particular experiment. Similar general physiological relations between the H-ion and streptococci, which are suggested by the above results, are also encountered in the results obtained in the other pH zones reported in Table V.

Table V.

Influence of H-ion Concentration upon the Relative Rate of  
Growth and Longevity of Different Types of Streptococci.

Broth of a series of different pH values received equal inocula of the different strains. Figures in table below represent the number of cells present after stated periods of incubation at 37° C.

Time (hrs.)	pH 5.5	pH 6.5	pH 7.5	pH 8.5
<b>Lactic</b> (Probable initial number of cells present;--250,000.)				
5	900,000	4,000,000	3,200,000	360,000
12	20,000,000	80,000,000	55,000,000	900,000
24	60,000,000	95,000,000	60,000,000	200,000
72	3,400,000	7,400,000	6,000,000	350,000
120	18,000	15,000	12,000	11,000
168	4,000	8,400	9,000	2,000
<b>Human</b> (Probable initial number of cells present;--90,000.)*				
5	50,000	750,000	1,200,000	190,000
10	500	1,400,000	3,200,000	250,000
15	20	1,900,000	1,500,000	140,000
24	-----	1,700,000	2,000,000	100,000
48	-----	800,000	1,400,000	12,500
72	-----	200,000	600,000	-----#
120	-----	1,600	3,000	700
168	-----	800	750	320
<b>Bovine</b> (Probable initial number of cells present;--100,000.)*				
5	200,000	900,000	1,500,000	150,000
10	500,000	2,000,000	2,600,000	180,000
24	5,200,000	5,400,000	9,500,000	100,000
48	3,000,000	2,300,000	4,000,000	160,000
120	-----#	18,000	12,000	1,000
168	-----#	10,000	16,000	300
<b>Cheese</b> (Probable initial number of cells present;--600,000.)*				
5	2,800,000	7,000,000	5,600,000	1,200,000
10	36,500,000	280,000,000	200,000,000	20,000,000
24	400,000,000	560,000,000	500,000,000	13,000,000
72	600,000,000	280,000,000	350,000,000	9,500,000
120	38,000,000	150,000,000	110,000,000	6,200,000
192	1,800,000	95,000,000	73,000,000	3,000,000

# Plates contaminated, but streptococcus colonies present;  
glucose broth tests, positive.

\* Recall footnotes, pp.

b. Influence of H-ion concentration upon the rate of growth and longevity of different types of streptococci.

The results given in Table V show the following general relations.

Zone showing maximum growth: The lactic strain exhibits its maximum rate of growth in media of a reaction representing a zone between pH 6.0 and pH 7.0. The maximum growth of a lactic strain in a similar zone has been reported by Itano from observations of turbidity, and more recently by Svanberg. The cheese strain also exhibits its maximum growth in this zone. The fact that this zone of most rapid growth for the lactic streptococcus approaches the pH value of milk has been pointed out by Itano. The human and bovine strains grow most rapidly in the zone between pH 7.0 and 8.0.

Comparative growth in limiting zones: In the zone representing pH values 8.0 to 9.0, the cheese strain is the only type which increased in numbers to a significant degree. Since this strain also exhibited the greatest acid tolerance in the experiment reported in Table IV, it is evident that this type possesses considerable resistance over a wide range of pH. Again, in the zone representing pH values 5.0 to 6.0, the cheese strain grows more rapidly than any of the other type strains.

The general significance of these relations and their comparison with the amino nitrogen increases in a similar series of pH values, will be given in the general discussion of the influence of H-ion concentration upon the different streptococci.

We wish to take this occasion, however, to point out the behavior of the human strain in broth of approximately pH 5.5. This streptococcus reaches a H-ion concentration of pH 5.0 as its

"fermentation limit". However, when inocula taken from cultures growing near the neutral point are introduced into plain broth of lower true acidity, they not only are not able to initiate growth, but rapidly decrease in numbers. The same relation is also shown by the bovine and lactic strains in the pH 4.5 test reported in Table IV. Hence, acidities less than their final "fermentation limit" appear to be not tolerable for the initiation of growth and, in fact, to lead to the death of the cells.

No extended discussion of these relations is warranted from our results. The distinction between the actual "fermentation limit" and the H-ion concentrations serving for the initiation of growth of the pneumococcus was shown by Avery and Cullen. The same distinction apparently exists in the case of streptococci. (The relation and distinction between the "fermentation limit" and the "limiting H-ion concentration for initiation of growth" have been discussed in detail in Part I of this paper.)

Relative longevity in different H-ion concentrations within the range of growth: The relation of H-ion concentration to the longevity of these strains cannot be interpreted directly from the above data. The rate of death in old cultures of any H-ion concentration probably follows a more or less orderly course. Consequently, the number of viable cells present at the time of any of the later analyses is dependent upon the number present in the period of maximum growth, as well as upon the H-ion concentration of the system. Moreover, in the above pH zones, in which growth has taken place at a variable and unequal rate, the concentration of metabolic products (upon which the rate of death is also dependent) probably is significantly different. Hence, there are too many factors involved in the determination of the numbers viable after long exposure to the different pH zones of growth, to permit the assignment of any specific effect to the hydrogen ion.



### 3. Summary and discussion of the influence of H-ion concentration upon the life processes of the different type strains.

#### a. Influence upon amino nitrogen increases.

The lactic strain splits peptone most actively in broth between pH 6.0 and 7.0. The cheese strain seems to require the same optimum zone for its most successful peptone attack, but its activity is inhibited to a much less extent than in the case of the lactic strain. The bovine and human strains prefer a pH zone 7.0 to 8.0 for peptolytic activity.

The increases in amino nitrogen evidenced in systems of different pH value are dependent upon several factors and the concentration of H-ion may exert a quantitatively different influence upon each of these factors. The influence of pH upon the growth of cells is one determinant of the concentration of the enzyme, and is thus involved in the determination of the rate of amino production. However, the final increase in amino nitrogen in systems of different pH value is more often a reflection of a number of simultaneous influences of the H-ion upon a number of other factors.

Chief among the factors so involved are the effect of the H-ion upon the activity of the enzyme or enzymes themselves, and the influence of the H-ion concentration upon the liberation of enzymes. Again, there is always a possibility of more than one enzyme being involved in the breaking down of protein derivatives, and the specific effect of the H-ion upon the members of such a possible enzyme complex may be decidedly different. (Bernby). It has also been shown that H-ion concentration exerts a significant influence upon the disintegration of bacterial cells and consequently, upon the liberation of co-enzymes. With these relations in mind, the assignment of a specific role to the H-ion in the determination of the amino nitrogen increases can not be approached without corresponding tests with enzyme solutions obtained from the strains involved.



Practical significance: The practical significance of the above results probably consists in the following relations. In systems of pH value approximately 5.5, the constituents of "peptone" are broken down more actively by the hemolytic cheese strain than by any of the other type strains. This suggests that members of this group may play a significant part in changes in the distribution of nitrogen in acid cheeses and in other dairy products, the H-ion concentration of which would inhibit the activity of true lactics to a greater extent.

b. Influence upon growth.

Optimum pH zones for growth: With the large pH increments tested in the above experiments, there was apparent agreement between the optimum zones for the splitting of peptone and for growth of cells.

Limiting initial H-ion concentrations: Each of the type strains proved unable to initiate growth in systems of lower true acidity than the final acidity reached in the glucose broth cultures of the same strain. Comparatively rapid death of the streptococci occurred in the zone which apparently lies between the usual "fermentation limit" and the "limiting H-ion concentration for initiation of growth".

Acid Tolerance: When introduced into broth pH 4.5 at 27° C., the type strains exhibit the following order of acid tolerance: cheese, lactic, bovine, and human. Altho this is the same order as in the case of their final H-ion concentration in glucose broth, it is evident that the so-called acid tolerance shows a

much greater divergence than would be expected from a comparison of the final pH reached in cultures of the same strain. Such a relation indicates that the final H-ion concentration or "fermentation limits" are not due merely to an intrinsic resistance to the bactericidal action of the H-ion.

Practical significance: The practical significance of these results lies in their projections to the illumination of the rôle of H-ion concentration in the determination of the moment of these different types of streptococci in the microbial balance in milk and milk products. These relations are important. An intelligent interpretation of the significance of the presence of different types of hemolytic streptococci in cheese and other dairy products, requires some knowledge of the resistance of these microorganisms and of their ability to grow in the acidities presented in acid milk products.

Given a sample of fresh milk in which all four of these types of streptococci were present, the more rapid rate of growth of the lactic and cheese types in systems of H-ion concentrations between pH 6.0 and 7.0, would explain the fact that they rapidly outgrow the human and bovine streptococci.\* The greater sensitivity of the human and mastitis strains to high acidities would tend to cause their disappearance in sour milk, butter and certain cheeses.

\*No intimation is made that the initial limiting pH zones are the same in milk as in plain broth. Avery and Culson have shown a decided difference in the H-ion concentrations permitting the initiation of growth of the pneumococcus in sugar-free and in sugar broths. However, it is doubtful if the relative rate of growth of the different types in different pH zones would be greatly disturbed by a change of medium. Nevertheless, the possibly selective influence of unknown factors in milk cannot be ignored.

The above suggestions are in keeping with common observations, such as those recently reported by Jones on the decrease in relative numbers of hemolytic udder streptococci in milk upon incubation. They are also in accord with the usual assumption that the hemolytic mastitis or udder streptococci are comparatively or relatively frequent in certified milk or in fresh market milk, but are outgrown by the common lactic in market milk at later periods of its handling and in fermented milk products.

The resistance of the cheese strain to high acidities would explain its appearance in significant numbers in milk products of high acidity, even though it were originally present in relatively small numbers.

## II. Influence of the Stage of Growth of the Culture

upon the Increases in Amino and Ammonia Nitrogen.

### 1. Experimental

The results in the preceding experiment suggested that a significant part of the increase in amino nitrogen occurred after the cessation of growth of the cultures. The present experiment attempts to compare the increases in amino nitrogen and ammonia nitrogen at different periods of the life history of cultures of streptococci.

#### Procedure: (Experiment 4.)

Inhibition broth, containing 0.2 per cent sodium phosphate was prepared in two lots of pH values 6.6 and 7.4. 100 cc. of the pH 6.6 broth was inoculated with the lactic strain; the same quantity of the pH 7.4 broth, with the human strain. These were incubated at 37° C. Samples were removed at the end of 1, 3, 5, and 10 days. Plate counts and  $\text{NH}_3\text{-N}$  determinations were made at each of these intervals.  $\text{NH}_3\text{-N}$  determinations were made from the 3 and 10 day samples.

Results are given in Table VI.

Table VI. a.

Influence of Stage of Growth upon Increases  
in Amino and in Ammonia Nitrogen.

Time days	NH <sub>2</sub> -N		NH <sub>3</sub> - N		Growth Stage
	mg.-in 100 cc.		mg.-in 100 cc.		Thousand cells in 1 cc. of culture.
Lactic	Total	Increase	Total	Increase	
0	55.1		9.8		
1	57.0	1.9			140,000.
3	58.5	3.4	16.5	6.7	80,000.
5	58.8	3.7			80.
10	59.5	4.4	16.7	6.9	4.
Human					
0	53.2		9.8		
1	56.2	3.0			6,000.00
3	58.6	5.4	16.3	6.5	2,000.00
5	60.4	7.2			8.00
10	62.0	8.8	17.1	7.3	.85

Table VI.b.

Comparison of Increases in Amino and Ammonia Nitrogen During  
Early and Later Periods of History of the Culture.

	Amino Nitrogen Increases		Ammonia Nitrogen Increases	
	First 3 days	3rd to 10th day	First 3 days	3rd to 10th day
Lactic	3.4	1.0	6.7	0.2
Human	5.4	3.4	6.5	0.8



# Relation of Stage of Growth of Cultures to Increases in $\text{NH}_2\text{-N}$ and $\text{NH}_3\text{-N}$ .

(Values plotted below are taken from Table IV.)

—  $\text{NH}_2\text{-N}$

● Human Strain

○ Lactic Strain

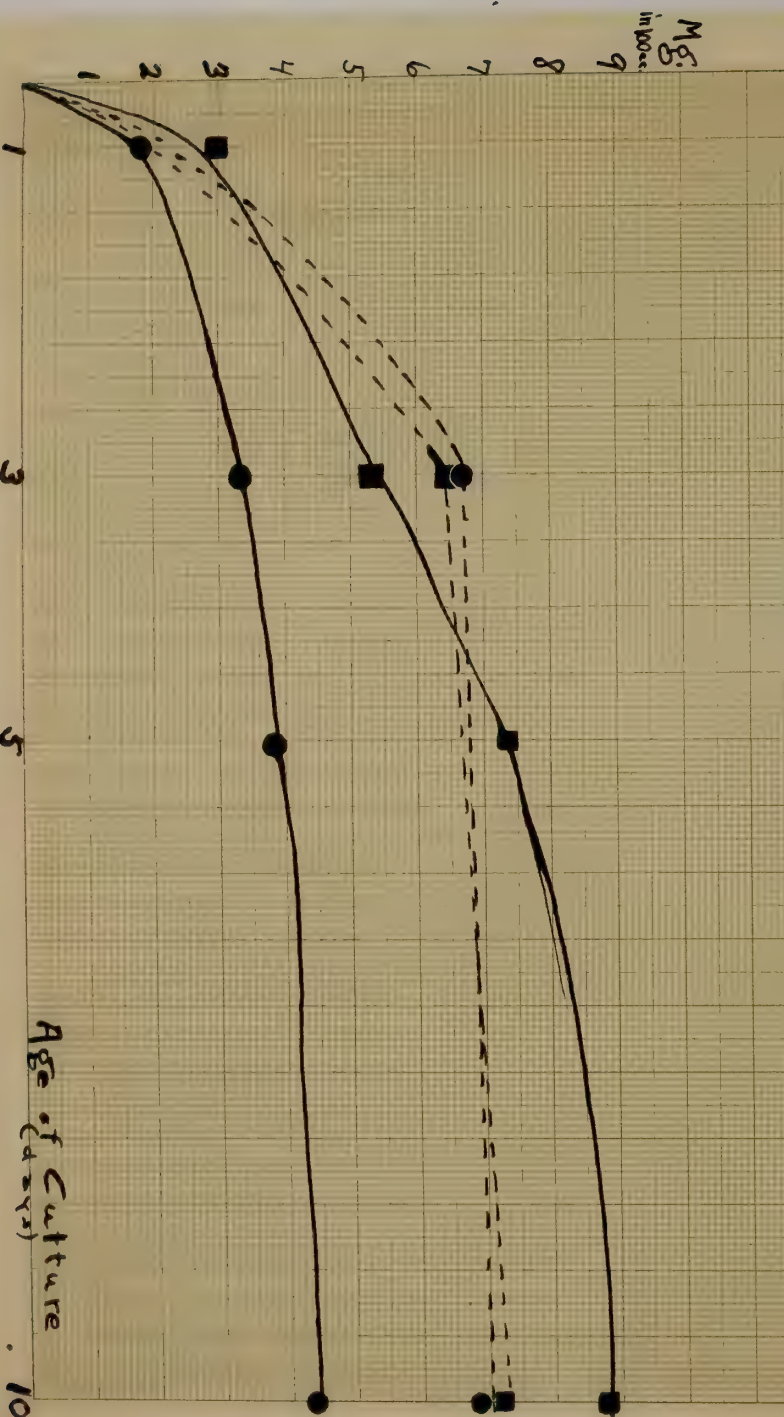


Figure 1.



It is evident in Table VIa that the cultures continue the formation of amino compounds considerably beyond the period of growth. In Table VIb it is shown that the increases in the amino nitrogen after the third day in the history of the culture represent a significant part of the total increase. During the same period, a much smaller proportion of the total increase in ammonia is formed than in the case of the amino nitrogen. This is also shown in Figure 1. A general discussion of the possible meaning of these relations will be given below.

## 2. General discussion of the relation of growth stage of cultures to increases in amino and ammonia nitrogen.

### a. Nature of nitrogen distribution in peptone broths.

As is well known, commercial peptone contains a greater variety of peptone derivatives than might be inferred from the use of the term "peptone". A certain amount of the total nitrogen is found in the form of compounds which are precipitated by the usual protein precipitants. This precipitated portion would be termed "protein nitrogen". In the same sample of peptone, the proportion of the total nitrogen to be termed "protein nitrogen" will vary with the precipitant used. (A. Hiller)\*. When precipitation is effected by colloidal iron, the "protein nitrogen" would consist of proteins and certain protein derivatives of only slightly less complexity (Van Slyke, Vinograd-Villichur, and Lossee). The filtrate would contain the nitrogenous constituents of lower complexity, and would be termed "non-protein nitrogen". This fraction would then be separated into "peptide", amino, and ammonia nitrogen. The

\*Unpublished manuscript by Van Slyke and Hiller.

peptide nitrogen would consist of that portion of the non-protein nitrogen which can be hydrolyzed to amino compounds. The amino nitrogen and ammonia nitrogen would consist of the portions of the non-protein fraction which can be determined as amino and ammonia compounds respectively.

In beef infusion peptone broth (culture medium used in this study) a further contribution to the total nitrogen is made by the infusion itself. A large part of its total nitrogen is in the form of amino compounds. (Infusion broth always contains a larger amount of amino nitrogen than does extract broth.)

The actual distribution of the nitrogen contained in peptone will, of course, vary with the degree of hydrolysis to which the product has been subjected in its manufacture. (It is a common observation that the usual American products contain a larger proportion of simple constituents than does the Witte product. Difco peptone used in this study, is perhaps one of the furthest hydrolyzed of the American products.)

#### b. Meaning of amino nitrogen and ammonia nitrogen increases in bacterial cultures.

From the above paragraphs it is seen that preformed amino and ammonia nitrogen exist in the culture medium before its inoculation. Both of these classes of nitrogenous substances are utilized as food by microorganisms. In the early period of the culture, the microorganisms are largely dependent upon the preformed simple nitrogenous constituents presented in the medium for the nitrogen portion of their food, whether used for energy or for growth. In all probability, the utilization of a certain amount of these substances precedes the actual formation of either. It is evident that both amino and ammonia compounds are utilized as well as formed by microorganisms, and that the utilization and formation of these compounds may proceed simultaneously and at constantly changing velocities.

With these facts in mind, it is obvious that determinations of either of these compounds, if made in the early history of cultures in media containing preformed amino and ammonia nitrogen, present more or less incidental values. Increases observed in their total concentrations merely represent the formation of a greater amount than has been consumed. The complexity of these relations is increased by the fact that the portion of amino nitrogen consumed may be taken either from that preformed in the system or from that which has been formed by the organism itself. From these relations, it follows that determinations made in the early history of cultures which do not actively and at an early stage attack peptone with the formation of amino and ammonia compounds, will show an actual decrease in the total concentration of these substances. Such findings are not infrequent. (No tests in the early periods of cultures have been made in this study. However, in Table IV I are presented results obtained with one strain which showed a slight decrease in amino nitrogen even after 10 days incubation; this strain did not show increases in amino nitrogen until cultures were several weeks old.)

In the above data, Table VIa, the increases in amino nitrogen in the 24-hour tests must be considered as representing the difference between the amount of amino nitrogen which has been formed and that which has been consumed in that period. The same probably applies to the 72-hour values but to a less extent as a decrease in the number of active cells is evident. The increases observed in the 5- and 15-day tests would seem to have occurred during a period in which little or no utilization of food would take place. The fact that the ammonia increases seem to be limited more closely to the earlier history of the culture suggests that ammonia production is more strictly

associated with the actual growth of streptococci.

The drawing of definite conclusions on the significance of these apparent relations is impossible in the face of the following possible complex sources, and methods of formation of amino and ammonia compounds in peptone broth cultures.

c. Possible sources and methods of formation of amino and ammonia compounds in peptone broth cultures of streptococci.

From the nature of nitrogen distribution in peptone broth, it follows that amino compounds may arise from the hydrolysis of a number of compounds of different nature. Both the peptide and protein fractions are capable of yielding amino acids,--moreover, amino nitrogen may be split off at the successive stages of their cleavage. It has been shown above that, during certain stages of the cultures, the utilization of amino compounds proceeds simultaneously with their formation. Hence, the utilization of amino compound may involve attacks upon the preformed amino nitrogen, or upon that formed by the organism or by its enzymes from any of the substrates which yield amino acids. Thus, the increase in amino nitrogen observed at any time represents the difference between the amount of amino compounds formed from any of the potential substrates and the amount of amino nitrogen consumed by the microorganism during the same period.

The production of amino acids may be assumed to be due to the hydrolytic action of the enzymes of the streptococci. However, many questions which are involved in the relation of the formation of amino nitrogen to the actual growth and metabolism of streptococci are unanswered today. The same is true of a satisfactory interpretation of the complete mechanism of its microbial formation. It does not seem that the formation of amino



acids can be considered merely as a waste product of microbial growth nor that their production is limited to periods in which growth is taking place. Neither does it seem that their production can be separated from the processes involved in the growth and life of the cells. However, it is probable that the total increases in amino nitrogen may be considered as including at least two general periods of formation, as based upon their relation to the growth of the culture. (It is understood, that such a division can be only approximate.)

A certain part of the total increase probably represents an unused residue of the total amount of amino compounds formed during the active growth of the culture. It is only reasonable to suppose that the living cell manifests at least a quantitative selection of amino acids from the collection presented to them (by the sum of the hydrolytic products of their enzymes and by those preformed in the system). The increase in amino nitrogen during the growth of the culture represents this unused residue, a part of which possibly can be considered as the portion less desirable to the organism, and a part merely as an excess production. These conditions would, of course, prevail only during the period of growth and activity of the cells themselves.

However, the above experiment shows that the production of amino compounds is by no means limited to the period of growth as the concentration of amino nitrogen increases during the period in which the cells are dying. The formation of amino compounds in this period may be regarded as due to the more or less incidental action of their "proteolytic" enzymes, whose activity often persists after the death and disintegration of the cells which elaborated



them. Hence, a certain part of the total production of amino acids is apparently due to the action of liberated enzymes,--more independent of the needs of the cell than in the former case. In this period probably all of the amino acids formed appear in the total increase of amino nitrogen in the system.

The production of ammonia by bacteria has been ascribed to the intra-cellular deaminization of nitrogenous food by the microorganisms (Kendall and Walker). This relation would indicate that ammonia formation is more closely associated with the growth and actual life of the cell than is the production of amine compounds. The results obtained in the above experiment are in keeping with this conception. Altho it is probable that the greater part of the total increase in ammonia is intimately associated with the metabolism of the cell, it is unfair to ignore the appearance of a small amount of ammonia as a possible cleavage product of the activity of hydrolytic enzymes.

### III. Influence of Temperature Upon the Peptolytic Action and Upon Other Life Processes of Different Types of Streptococci.

The influence of temperature as a factor in the environment of lactic acid bacteria has been reviewed in detail in Part I of this paper. There it was shown that temperature may be a conditioning factor of the rate, the extent, and at times, the direction of microbial processes. ("Influence of Environment upon the Lactic Acid Bacteria"; "Theoretical Progress of Lactic Acid Fermentation".)

With these relations in mind, a study was made of the relative influence of different temperatures upon the life processes of the different types of streptococci. The pertinence of such a study

is directly evident from the significance of temperature in the systems in which these different streptococci are found. The direct significance of these temperatures upon the different types of streptococci will be discussed in the interpretation of the results of the present study.

# 1. Influence of different temperatures upon amino nitrogen increases.

The influence of different temperatures upon the amino nitrogen increases effected by the different types of streptococci was studied by comparing the changes brought about by the type strains in the same system under different temperature conditions. The temperatures of 15°, 25°, 31°, and 41° C., were chosen as test temperatures.

As a rough index of the influence of temperature upon the rate of peptone splitting, determinations were made of the amino nitrogen increases brought about by equal inocula after 24 hours incubation at different temperatures. As a means of comparing the influence of temperature upon the final increase in peptolytic products, determinations were made of the final increases in amino nitrogen after longer periods of incubation.

## Procedure: (Experiment 5)

Infusion broth, pH 7.2, containing 0.2% sodium phosphate, was sterilized in 100 cc. portions. Series of flasks of medium were placed in constant temperature rooms at 15° and 25° C.; other series were placed in incubators at 32° and 41° C., respectively. The media were held over night at these temperatures before inoculations were made.

Flasks of the different series received equal inocula of the respective strains, and were then incubated at the designated temperatures. The 25° series varied in temperature from 22-25° C.; the 15° series, from 14-15° C.  $\text{NH}_3\text{-N}$  determinations were made from 1, 5, and 15 day samples. Results are given in Table VII.

Table V II.

Amino Nitrogen Increases at Different Temperatures.

Medium: Infusion broth, pH 7.2, containing 0.2 per cent sodium phosphate. Flasks of each temperature series received equal inocula of the respective strains. Results expressed below as mg.  $\text{NH}_2\text{-N}$  in 100 cc.

	1 day	5 days	15 days	Increase in $\text{NH}_2\text{-N}$ .
Human				
15°	----#	55.4	55.5	---
23°	56.4	63.9	65.0	9.5
31°	57.9	64.1	65.4	9.9
41°	55.9	58.2	58.5	3.0
Bovine				
15°	----#	55.5	55.5	---
23°	57.0	57.5	58.7	3.2
31°	58.9	59.1	59.4	3.9
41°	58.8	60.0	60.1	4.7
Cheese				
15°	55.0	61.4	62.0	6.5
23°	60.0	63.0	64.3	8.8
31°	63.7	64.0	64.8	9.3
41°	63.9	64.8	65.3	9.8
Lactic o				
15	54.7	58.9	60.2	4.7
23°	57.8	59.1	60.6	5.1
31°	58.6	59.5	60.3	4.8
41°	57.5	58.6	58.8	3.3

# Not determined.

The results given in Table VII show the following general relations.

Peptone splitting by the lactic strain proceeded most rapidly in the temperature zone approximating  $30^{\circ}$  C. Although the greatest rate is exhibited at this temperature, the final increase in amino nitrogen is greatest at a somewhat lower temperature. The human and bovine strains seem to split peptone most rapidly at temperatures between  $30$  and  $40^{\circ}$  C. The cheese strain, however, shows a greater rate of increase at a temperature of  $41^{\circ}$  than at  $31^{\circ}$  C.

The final increase in amino nitrogen is greatest in the  $31^{\circ}$  test in the case of the human strain. Significant increases occurred in the greatest range of temperatures in the case of the cheese strain, although the lactic strain is relatively more active at the lower temperatures. At a temperature of  $41^{\circ}$  C., the processes involved in the production of amino compounds are inhibited to a marked degree in the case of the human and lactic strains. This inhibition is exhibited both in the rate of peptolysis and in the final increase in peptolytic products. The bovine and cheese strains seem to exhibit their greatest increases in the  $41^{\circ}$  test. (This phenomenon is explained in the next experiment.)

The significance of these relations will be discussed in the general review of our study of the influence of temperature upon the life processes of different types of streptococci.

## 2. Comparison of amino nitrogen increases at $37^{\circ}$ and $41^{\circ}$ C.

The bovine and cheese strains had exhibited greater amino nitrogen increases at  $41^{\circ}$  than at  $31^{\circ}$  C., in the above experiment. While it did not seem probable that these strains would be more

active at temperatures slightly above  $37^{\circ}$  C. than at the usual incubation temperature, the possibility of a higher optimum temperature could not be ignored. Such a temperature relation would be peculiarly pertinent in the case of the bovine or mastitis strain as the usual body temperature of cows is higher than that of humans. To investigate such a possible relation, the following experiment was conducted:

Procedure: (Experiment 6)

Two series of flasks of 30 cc. of infusion broth (pH 7.2 and containing 0.2 per cent sodium phosphate), received inocula of 0.1 cc. of the human, bovine, and cheese strains. The series were incubated at the temperatures of  $37^{\circ}$  and  $41^{\circ}$  C., respectively.  $\text{NH}_2\text{-N}$  determinations were made at the end of 7 days incubation. results are given below in Table VIII.

Table VIII.

Comparison of Amino Nitrogen Increases at  $37^{\circ}$  and  $41^{\circ}$  C.

Medium: Infusion broth, pH 7.2, containing 0.2 per cent sodium phosphate.  
Cultures 7 days old at time of analysis.

	Total $\text{NH}_2\text{-N}$ .		Increase in $\text{NH}_2\text{-N}$ .	
	mg. in 100 cc.		mg. in 100 cc.	
	$37^{\circ}$	$41^{\circ}$	$37^{\circ}$	$41^{\circ}$
Human	64.5	61.0	9.2	5.7
Bovine	59.0	58.6	3.7	3.3
Cheese	64.8	64.5	9.5	9.2
Control	55.3	55.3	---	---



The answer to the primary question involved in this experiment is evident in the results given in Table VIII. The total increases in amino nitrogen are less at  $41^{\circ}$  than at  $37^{\circ}$  C. in the case of all of the type strains. Hence, this temperature is above the optimum for the cheese and bovine strains. As in the preceding experiment the higher temperature does not inhibit their peptone attack to so marked a degree as in the case of the human strain.

It will be observed in Table VII that the bovine strain exhibited a greater amino nitrogen increase at  $41^{\circ}$  than at  $31^{\circ}$  C., while in Table VIII the increase is greater at  $37^{\circ}$  than at  $41^{\circ}$  C. The explanation of these relations is obvious. The temperature of  $37^{\circ}$  is apparently more near the optimum than is  $41^{\circ}$  C. This higher temperature depresses the peptolytic action to some extent, altho' even at this temperature, the bovine streptococcus is more active than at  $31^{\circ}$  C., a temperature considerably below its optimum.

It will also be observed that in this experiment the  $41^{\circ}$  test of the human strain is depressed to a greater extent than in the  $41^{\circ}$  test reported in Table VII. This depression happens to displace the order of increase among the different types. However, the same general temperature influence is still evident (the bovine and cheese strains are inhibited to a less extent at  $41^{\circ}$  than is the human strain). In the following experiment an investigation is made of one of the factors involved in the determination of the activity of the human strain at temperatures above  $37^{\circ}$  C. as a probable explanation of such observations.

### 3. Influence of size of inoculum upon amino nitrogen

increases at optimum temperature, and at temperatures above the optimum.

In the several tests made of the amino nitrogen increases at  $41^{\circ}$  C., discrepancies frequently were evident. This was especially true in the case of the human strain (see above statements). In a search for the factors involved in the apparently irregular growth and peptone attack at this temperature, tests were made using different sizes of inocula. Tests made at  $41^{\circ}$  with inocula of different sizes frequently showed evident differences in the amount of growth, even when incubation was continued until cultures were sterile. Similar tests made at

37° C. showed no apparent difference. The following experiment is a report on one of these tests.

Procedure: (Experiment 7)

Infusion broth, pH 7.4, containing 0.2 per cent sodium phosphate, was used as the medium. Three flasks, each containing 60 cc. of broth, were placed in a water bath at 41° C. for 12 hours. They were then inoculated with the following amounts of a 12-hour culture of the human streptococcus: flask 1, one loopful; flask 2, two loopfuls; flask 3, 0.2 cc. This series was then immersed in a water bath held at 41° C. inside of an incubator, and incubated for nine days. The temperature did not vary more than 0.2° during the experiment. The tests were kept immersed so that the surface of the medium was at least an inch below the surface of the bath.

Two flasks of the same medium were inoculated with one loopful and 0.2 cc. of the culture used above, and incubated at 37° C. for the same period.

Observations of growth were made daily for four days, after which time all members of the 41° series were sterile.  $\text{NH}_2\text{-N}$  determinations were made at the end of nine days. Results are given in Table IX.

Table IX.

Influence of Size of Inoculum upon Amino Nitrogen Increases at Optimum Temperature and at Temperatures Above the Optimum, in Cultures of the Human Hemolytic Streptococcus.

Medium: Infusion broth, pH 7.4. Tests received inocula of sizes indicated below. The 41° series were sterile after 4 days.  $\text{NH}_2\text{-N}$  determinations were made at the end of 9 days.

Amino Nitrogen

mg. in 100 cc.

	41°	37°
Inocula		
Control	53.0	53.0
1 loop	53.3	62.6
2 loops	53.6	
0.2 cc.	56.5	62.7

The results given in Table IX show that the amino nitrogen increases by this strain of streptococcus are severely conditioned by the size of the inoculum, at the temperature of  $41^{\circ}$  C. At the optimum temperature the final increase in amino nitrogen does not seem to be dependent upon the size of the inoculum, within the limits tested.

Reports on the influence of the size of the inoculum upon bacterial growth are frequent in the literature. Many of these are apparently limited to the ability to initiate growth, as in the case of Cole's experiments with the pneumococcus\*. Studies on the rôle of accessory food substances in bacterial growth and nutrition furnish examples of similar phenomena, together with at least a partial explanation of many of the earlier observations.\* With these reports the present paper is not concerned.

Here, it is merely desired to point out that at temperatures above the optimum, the luxuriant growth of microorganisms may exhibit much greater dependence upon the size of the inoculum, than at optimum temperatures. This, of course, is to be expected but the factors involved should not be discussed without a greater amount of experimental evidence.

In a number of our  $41^{\circ}$  tests, the influence of the size of the inoculum does not seem to be limited to the ability to initiate growth. Frequently, small inocula gave slight growth but never approached the growth evident in tests of the same series with large inocula. This obtained even when cultures were incubated until absolutely sterile.

\* Personal communication of unpublished work, by Dr. O.T. Avery.

#### 4. Influence of temperatures above the optimum upon the final H-ion concentration in glucose broth.

In several tests made in conjunction with the preceding experiments, the ability of the different strains to grow at 41° C. was controlled by culture in glucose broth. In one of these tests the pH of a glucose broth culture of the lactic strain was roughly tested by the addition of several drops of methyl red. This culture gave a salmon yellow color, which indicated that lower final H-ion concentrations were attained at temperatures above the optimum.

It seemed desirable to study the influence of such temperatures upon the final H-ion concentrations reached by the different type strains. The results obtained would be of value in the interpretation of the general influence of high temperatures upon the life processes of the different streptococci. They would also represent a small contribution to our knowledge of the various environmental factors which may at times obscure the specific effect of the H-ion.

#### Procedure: (Experiment 8)

Medium: Glucose infusion broth. 50 cc. portions of the medium were brought to a temperature of 41° C. They were then inoculated with 0.3 cc. of a 12-hour culture of the respective strains. The inoculated flasks were placed in the 41° water bath used in the previous experiment.

Another series received the same inocula and was incubated at 37° C.

After 5 days incubation electrometric determinations of the final pH were made by Dr. A. Itano. It is believed that these figures represent final values as all of the 41° test cultures were sterile at the time of pH determinations with exception of the cheese strain. This culture contained comparatively few viable cells by the plate method.

The results are given in Table X.



Table X.

Comparison of Final H-ion Concentrations of  
Glucose Broth Cultures at 37° and at 41° C.

Medium: glucose infusion broth, p H 7.4. Inocula: 0.3 cc. of 12-hour cultures, in 50 cc. of test medium. Cultures 5 days old; human, bovine, and lactic cultures of the 41° series were sterile at the time of pH determinations.

	Human	Bovine	Lactic	Cheese
41°	5.6	4.7	5.0	4.3
37°	5.0	4.4	4.2	4.1

The results in Table X are believed to represent final values with the possible exception of the cheese strain. The fact that the human, bovine, and lactic cultures were sterile at the time of the test, requires that any further acid production be due to purely enzymatic reactions. The well known sensitivity of lactic acid producing enzymes renders it improbable that these enzymes of the streptococci are more resistant to exposure to high H-ion concentrations at 41°, than are the cells themselves. The work of Avery and Cullen on the enzymes of pneumococcus (a close relative of the streptococci) has shown that its enzymes are relatively sensitive to deleterious influences.

If these figures are final values, and there is little reason to believe they are not, the final H-ion concentration reached in glucose broth at temperatures above the optimum are significantly different than those obtained at the usual incubation temperatures. The difference is especially evident in the case of the human and lactic strains which throughout our study have proven relatively more susceptible to high incubation temperatures than have the bovine and cheese strains. There is nothing surprising in the above findings, altho' similar examples in case of streptococci have not been given in the literature. It is especially interesting, to note that the lactic strain would be "methyl red negative" at this temperature.



The importance of the above results to the relation of H-ion concentration to general microbiology is as further evidence that other factors often obscure the specific effect of the H-ion. Recent literature has offered numbers of examples in support of Clark's early contention that the "physiological constant" conception of the final H-concentration requires a strict definition of the fermentation system.

The lower "fermentation limit" reached at temperatures above the optimum may be explained most easily by the

$$\text{Speed} = \frac{\text{Driving Force}}{\text{Resistance}}$$

formula used by Getman to explain the end points or final equilibria of catalytic reactions. It is only reasonable to suppose that the "resistance" at 41° is greater than at the optimum temperature. Hence, the product of all of the factors involved in the final inhibition of acid production, would reach a value reducing the "speed" to zero at a H-ion concentration which in itself would be insufficient to prohibit further acid production at the optimum temperature. Again, a positive temperature coefficient for the product of all of these forces which are involved in the final inhibition of growth of bacterial cultures, would also require a lower final acidity at a higher temperature.

The factors conditioning the "fermentation limit" of microorganisms are discussed in detail in Part I of this paper. (See "Influence of H-ion Concentration" under "Influence of the Environment of Lactic Acid Bacteria"; also, "End Point of the Lactic Acid Fermentation Process" under "Theoretical Progress of Lactic Acid Fermentation".)

Fairly large inocula were used in the above tests. Whether the size of the inoculum also influences the final pH at 41°, is not known. A study of this factor was not made as the direct object of this experiment was merely the comparison of the relative influence of higher incubation temperatures upon the life processes of the different types of streptococci.

5. Influence of temperature upon the rate of growth and upon the activity and viability of different types of streptococci.

The influence of temperature upon the products of microbial activity has been studied in the preceding experiments. On last analysis, the influence of temperature upon the accumulation of the products of microbial processes is in many cases, at least in part, a reflection of the influence of those temperatures upon the sum total of all of those processes which are involved in the actual growth of the cells. The relation, however, is not necessarily parallel, nor always direct. The accumulation of certain products is not due entirely to the actual growth of the cells, as was suggested by the results obtained in Experiment 3 (Table VI).

For the above reasons it was desired to compare the direct influence of different temperatures upon the growth and multiplication of the different streptococci. It was also desired to compare the general vitality of cultures of the different types, which had been incubated at different temperatures for equal periods of time. The continuation of such a comparison to longer periods of exposure offered a means of comparing the relative longevity of the different types after equal time exposures to different temperatures. The following experiment was conducted in the study of these relations.

## Procedure: (Experiment 9).

The experiment has the following direct objects:

- (1) An approximate comparison of the relative rate of growth of the different types of streptococci at different temperatures.
- (2) A comparison of the "general growth condition" and vitality of cultures of equal age which have been growing at different temperatures; this extends itself to a comparison of the relative longevity of the different streptococci after equal time exposures to different temperatures.

### a. (Object I.)

relative rate of growth of the different streptococci at different temperatures.

The satisfaction of Object I required an approximation of the relative rate of growth in cultures incubated at different temperatures. The procedure employed to meet this end involved the estimation of the relative number of cells present in the various members of a series of broth cultures which had received equal inocula of the respective strains and which had then been incubated for an equal period of time. This set of conditions suggested the use of the method described as the "Andrade test" in the statement of "Methods of Study". In the approximate comparison of numbers, this method has been limited to tests made with young cultures; it also requires that no member of the test series has been exposed to previous unfavorable environments. This set of conditions will obtain in young cultures of the 15°, 25°, and 32°, members of the temperature series. It is not met, however, in the 41° test as there is a strong probability that inocula taken even from young cultures grown at temperatures above the optimum cannot be compared with those taken from cultures held at lower temperatures.

### b. (Object II.)

Comparison of the relative activity and vitality of cultures of the different types of streptococci when incubated at different temperatures.

The satisfaction of Object II requires an approximation of the "general growth condition" or vitality of the different cultures. The principles involved in such an approximation have been developed in the description of the "Andrade test". ("General Methods of Study"). From the relations discussed in that place, it is evident that the conditions underlying

the satisfaction of Object II are met by the second set of conditions described in our former explanation of the above method.

The relative rate of acid production of inocula taken from these cultures will be an approximate function of the relative growth condition and vitality of the various cultures under comparison. Here, one is not limited to the comparison of young cultures nor to temperatures not above the optimum. In fact, the actual object in mind is the relative comparison of the influence of all of the various conditions in the environments obtaining at different temperatures. For this reason the use of the above described method seems to be justified, in an approximate estimation of the relative influence of different temperatures upon the vitality of the different types of streptococci.

Object II also requires the comparison of the relative longevity of the cultures at different temperatures. The above method, of course, is essentially a test of the presence of viable cells. However, for actual determinations of sterility larger samples must be taken as test inocula.

#### Detailed procedure:

The temperature series were prepared as described in Experiment 5; equal volumes of broth received equal inocula of the respective type strains; these cultures were incubated at the temperatures of 15°, 23°, 31°, and 41°, respectively. (The test media were maintained at the proper temperatures previous to, and during inoculation.)

The detailed manipulation involved in the "Andrade test" has been given in the description of the method. (General Methods of Study). One cc. samples were removed from each member of the series; the sample was diluted in sterile salt solution; 1.0 cc. of the dilution (representing 0.01 cc. of the original sample) was introduced into uniform test tubes containing 12 cc. of sterile glucose Andrade infusion broth. These tests were incubated at 37° C., and observations made of the time required for the attainment of a distinct pink color. Tests were made in duplicate. The 1 day test period was used in the approximation of the rate of growth. The 3, 5, and 15 day tests were used in the comparison of the general growth condition and vitality of the cultures.

For a check on the possible sterility of cultures, undiluted 1.0 cc. samples were introduced into glucose broth. This test was made at each of the time periods in the case of the 41° test series, and at the 15 day period with all of the cultures.

The data obtained are given below in Table XI; they are restated in Tables XII and XIII.



Table XI.

Data Obtained in Experiment 9.

Hours Required for Acid Production by Inocula Taken  
From Cultures Incubated at Different Temperatures.

Temperature test series received equal inocula of cultures of each type. These cultures were incubated at different temperatures. Figures below represent time required for production of equal amounts of acid by equal samples taken from members of the above temperature series after stated periods of incubation.

Age of Culture. days		15°	23°	32°	41°
Lactic	1	9	6½	6¼	9
	3	7½	7¼	8	36
	5	8	8	8½	sterile
	15	14½	16	18	-
Human	1	19	11	10¼	32
	3	16	7¾	8½	sterile#
	5	18	11	12¼	sterile
	15	24-36	24-36	24-36	-
Bovine	1	13	7½	6¾	9½
	3	12¾	6	6¼	11
	5	13	6½	7¼	13
	15	14	9¾	10½	sterile
Cheese	1	10	6	6	6
	3	7½	6	6	6½
	5	6½	6½	7	8½
	15	7¼	8¼	10	12¾

# .01 cc. sterile; 1 cc. positive in sterility tests made in glucose broth.



The data reported as a whole in Table II can be used to better advantage in the interpretation of the problems in mind by an independent attack upon each of the objects of the experiment.

### a. (Object I.)

Relative rate of growth of the different streptococci at different temperatures.

The use of the data in Table III in this connection is limited. For reasons stated above, none of the figures obtained in the 41° tests can be interpreted here. Altho' 24-hour broth cultures of streptococci are not young cultures when incubated at the usual incubation temperatures, there should be no serious objection to the use of cultures of this age which have been grown at the lower temperatures of this series.

Hence, it seems that these figures should serve for an approximate comparison of the relative influence of different temperatures upon the rate of growth of the different type strains. With this interpretation the 24-hour values reported in Table III are restated below.

Table III.

### Influence of Different Temperatures Upon the Rate of Growth of Different Types of Streptococci.

Relative rate of growth at different temperatures, based upon the comparative rate of acid production of equal incubations from cultures which had been incubated 24 hours at different temperatures.

In the table below are presented the reciprocals of the 15°, 23°, and 37° values given in Table II--i. e., these figures represent the reciprocals of the number of hours required for the production of equal amounts of acid by equal incubations of cultures which had been incubated 24 hrs. at the stated temperatures. These reciprocal values do not have a common basis, and are related only to the values obtained for the same strains.

	15°	23°	37°
Lactic	.11	.15	.16
Human	.05	.09	.10
Sovine	.07	.13	.15
Cheese	.10	.17	.17

The following general relations are evident in Table XII.

The lactic streptococcus exhibited its most rapid growth in the temperature series approximating  $30^{\circ}$ . Its rate of growth at  $15^{\circ}$  is relatively greater than that of any of the other types. The human and bovine strains also show more rapid growth at  $30^{\circ}$  than at lower temperatures. At  $41^{\circ}$  the growth of the human strain is inhibited to a more marked degree than is that of the mastitis strain.

The figures obtained for the cheese strain are of little meaning in the direct interpretation of the influence of temperature upon the rate of growth. Apparently, the cheese type had reached its maximum growth in 24 hours at all temperatures except the lowest member of the series. With the resistance peculiar to this strain, the activity of the cultures at these temperatures did not rapidly diminish after the attainment of maximum growth.

b. (Object II.)

Comparison of the relative activity and vitality of cultures of the different types of streptococci when incubated at different temperatures.

For reasons given above in the preceding discussion, it seems that all of the data presented in Table XI can be used in comparing the relative influence of different temperatures upon the general activity and vitality of the different streptococci. To serve as a means of more ready comparison of the figures obtained, they are presented in a slightly different form in Table XII.

In the case of each strain, the test which showed the most rapid acid production is assumed to represent the temperature-time period in which that strain exhibited its greatest activity. This assumption is of course limited to the temperature-time periods tested. A larger number of tests would be required for the "period of greatest activity" so obtained, to represent anything approaching an absolute value.

In the presentation below, (Table XIII), the temperature-time "period of greatest activity" of the different strains is expressed as unity. The reciprocals of the time required in the other tests of each strain is then multiplied by the time required in the test taken from the temperature-time "period of greatest activity" of the same strain. The relation between these figures is by no means absolute or definite. Nevertheless, there is reason to believe that they may serve as convenient indices of the influence of different temperatures upon the general activity and vitality of the different streptococci. These figures are given in the following table. (Table XIII).

Table XIII.

Relative Activity and Vitality of Cultures of Different Types of  
Streptococci Grown at Different Temperatures.

(See text for limitation of values reported below, and for the basis of their assignment).

	Age of Culture. days	15°	23°	32°	41°
Lactic	1	.69	.93	1.00	.69
	3	.83	.81	.78	.18
	5	.78	.78	.74	---
	15	.44	.39	.35	---
Human	1	.41	.70	.76	.26
	3	.49	1.00	.92	---
	5	.43	.70	.64	---
Bovine	1	.46	.80	.89	.63
	3	.47	1.00	.92	.55
	5	.46	.92	.83	.43
	15	.43	.62	.57	---
Cheese	1	.60	1.00	1.00	1.00
	3	.80	1.00	1.00	1.00
	5	.92	.92	.86	.71
	15	.83	.73	.60	.47

The values given in Table XIII show the following general relations:

Growth of the lactic strain is apparently inhibited to a less extent by low temperatures than is that of the other type strains. The human strain exhibited the narrowest range of temperature for growth.

Both the lactic and the human streptococci, while able to grow to some extent at  $41^{\circ}$  C., are rapidly weakened and killed after comparatively short exposure to that temperature. As in previous experiments at  $41^{\circ}$ , the life processes of the cheese and mastitis strains are inhibited to a less extent.

The resistance of the cheese strain at this temperature was striking as a survival of 15 days at  $41^{\circ}$  C. is a surprising exhibition for a hemolytic streptococcus. However, this strain also proved exceedingly resistant to all other unfavorable environmental conditions. On the other hand, the relative resistance of the mastitis strain at  $41^{\circ}$  is more interesting. Its resistance relative to the human strain is easily explained by assuming the human streptococcus to be in general a more delicate organism. However, the greater resistance of the mastitis strain in comparison to the lactic streptococcus cannot be explained upon a similar basis.

It was found that the bovine strain was less resistant in heating experiments conducted at temperatures above  $60^{\circ}$  C. (In tests made in milk, the lactic survived the conditions of the pasteurization process, while the mastitis strain quickly succumbed.) While these two experiments are not directly comparable, it is evident that the mastitis streptococcus is not more resistant to a given period of exposure to temperatures above  $60^{\circ}$  C. Such a relation might point to a possibility of differences in the temperature coefficients of the disinfection rates of these two strains between  $41^{\circ}$  and  $63^{\circ}$  C. The question



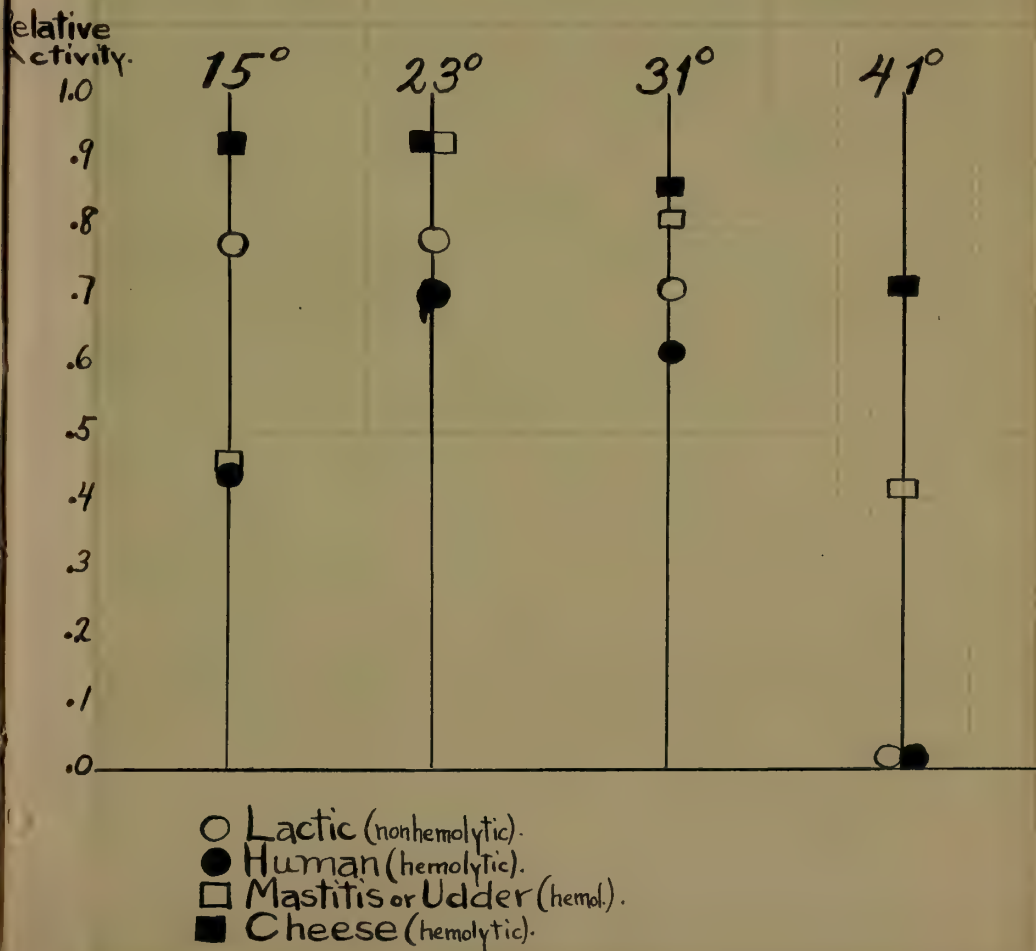
however, is obscured by the probable influence of previous culture upon the resistance of both strains to heat. In the absence of further experimental data, it is more wise to assume that the difference in longevity of the bovine and lactic strains at  $41^{\circ}$  C., represents a relation dependent upon the fact that this temperature is further removed from the optimum temperature for growth of the lactic strains.

The relative activity of cultures which, after receiving equal inocula of each type strain, were incubated for 5 days, may be used to illustrate the general behavior of the different type strains in environments of different temperatures. These 5 day values are plotted below in Figure 1. This figure is well adapted to an immediate survey of the general relations of the type strains to the different test temperatures.

The following relations are among those evident in Figure 2. The  $15^{\circ}$  values for the human and bovine strains represent cultures which have not multiplied since the initial inoculum (compare values given in Table XIII). The cheese and lactic strains, which grew at this temperature, have diminished in activity to a less extent at  $15^{\circ}$  than at the higher temperatures. The well known greater longevity and more persistent activity of cultures held at lower temperatures is evident in all of the series in the figure, and requires no further discussion. The sudden drop in activity of the  $41^{\circ}$  tests of the human and lactic strains is indicative of the sensitivity of these strains to temperatures above  $37^{\circ}$  C.

# Relative Activity of Cultures of Different Types of Streptococci, which Have Been Incubated 5 Days at Different Temperatures.

(values plotted below are taken from Table XIII.)



## 6. Summary and discussion of the influence of temperature upon the life processes of the different streptococci.

### Influence upon amino nitrogen increases.

The lactic strain exhibits greater relative activity at temperatures below 30° than do any of the other type strains. The lactic streptococcus produced a higher final concentration of amino compounds at a temperature lower than that required for greatest rate of formation of amino compounds. This is not an unusual observation as frequently higher final end points are attained at temperatures below that at which the greatest velocity is displayed. (See "Theoretical Progress of Lactic Acid Fermentation" - Part I of this paper).

At 41° C., the peptone splitting powers of the bovine and the cheese strains are inhibited to a less extent than are those of the lactic and the human strains.

### Influence upon final H-ion concentration.

At 41° C. lower final true acidities are reached in glucose broth by the different type strains than at 37°. As in the case of the amino nitrogen production, the inhibition of the bovine and cheese strains is relatively less than that of the human and lactic types.

### Influence upon growth and activity.

Much the same is the influence of temperature upon the rate of growth as upon the rate of amino production. As is to be expected, the decrease in activity is less at lower temperatures than at high temperatures in the case of all strains. The bovine

and cheese strains not only grow more actively at 41° than do the other types, but also decrease in activity less rapidly at this temperature.

In the case of the bovine strain, it does not seem that its relative resistance to this temperature is due to an intrinsic resistance to disinfection by heat. It seems more probable that 41° merely represents a point nearer the optimum temperature of the bovine than of the lactic strain, rather than that the bovine streptococcus is more resistant to a given period of exposure to high temperatures.

#### Practical significance.

The practical significance of these temperature relations consists in the relation of temperature to the determination of the relative number of different types of streptococci present in milk and milk products held at different temperatures. The human and mastitis strains (in addition to the before mentioned influence of H-ion concentration) would be outgrown in milk held at low temperatures by the lactic<sup>and</sup> cheese strains.

It is also evident that both the cheese and lactic strains could attack the nitrogenous constituents of cheese at low temperatures.

#### IV. Influence of Oxygen Concentration upon Amino Nitrogen Increases.

The influence of oxygen concentration as a factor in the environment of lactic acid bacteria has been reviewed in detail in Part I of this paper. ("Influence of Environment upon Lactic Acid Bacteria".) There it was shown that this factor plays an



important rôle in the determination of many microbial processes. The importance of oxidation processes as a means of obtaining energy for microbial growth has also been discussed in detail in part I. ("Chemical Changes Involved in Lactic Acid Fermentation"- "Transformation of Energy".) The general significance of an investigation of the influence of oxygen concentration upon the activity of the different types of streptococci is evident by a review of those discussions.

The direct significance of a comparison of the influence of different oxygen concentrations upon the peptone attack of the different streptococci, lies in the fact that these different types of streptococci frequently are found at different times in systems of widely different oxygen concentrations. The "human" and "bovine" streptococci are commonly found in the animal body under conditions, possibly differing in oxygen concentration. Lactic streptococci are also found under wide ranges of oxygen concentration. The concentration of oxygen in milk is rapidly changing during microbial growth, and as Marshall\* has shown, soon approaches a minimum. In cheese and other systems in which the lactic group are involved, the concentration of oxygen may also be a factor in determining the rate, the extent, or possibly the direction of the changes brought about by lactic acid bacteria.

The importance of all of these relations pointed to the pertinence of the study of the relation of oxygen concentration to the peptone attack of the different types of streptococci. The investigation of these relations consisted in the comparison

\*Personal communication.



of the amine nitrogen increases effected by the different streptococci, when grown in systems differing only in the concentration of atmospheric gases.

**Procedure: (Experiment 10).**

Anaerobic conditions were obtained by the method suggested by the recent work of Bates and Olitsky. The principles of the method consist in deoxygenation of the medium by boiling and overlaying the deoxygenated medium with sterile melted vaseline. The melted vaseline serves as an effective seal inhibiting the access of oxygen. Anaerobic conditions are maintained by the reducing action of peptone in faintly alkaline systems at a temperature of  $37^{\circ}\text{C}$ . Methylene blue was used as the indicator of the effectiveness of the removal of oxygen and of the maintenance of deoxygenation.

Detailed procedure: 50 cc. of infusion broth, pH 7.3, was sterilized in the tubes commonly used in the "reductase" test in milk analysis. 0.4 cc. of a 1 per cent sterile solution of methylene blue was added to one tube to serve as a later check on the anaerobic conditions. The tubes and check were placed in the steamer at  $100^{\circ}\text{C}$ . and boiled until decolorization of the check indicated that the dissolved air was driven out of the medium. The tubes were then removed separately, and the medium overlaid with sterile melted vaseline and immediately cooled in running water to solidify the vaseline. The same manipulation was employed with the check. The methylene blue remained decolorized during the time required for the overlaying of the vaseline, which proves that appreciable amounts of oxygen did not enter the medium during this procedure. The tests and the check were then incubated for 72 hours at  $37^{\circ}\text{C}$ . to ensure sterility and anaerobic conditions.

The same broth was used in the case of the aerobic series. In this case, 50 cc. portions were sterilized in Warlenmeyer flasks and subjected to the same heating conditions as the anaerobic series.

The two series received equal inocula of each strain. The anaerobic series were inoculated by means of a capillary pipette. The lactic tests were incubated at  $32^{\circ}\text{C}$ ; the other strains, at  $37^{\circ}\text{C}$ . After 8 days incubation, HNE-N determinations were made. Results are given in Table IV.

Table XV.

Influence of Oxygen Concentration upon Increases in Amino Nitrogen.

Medium: Infusion broth, pH 7.3. Anaerobic conditions were maintained by vaseline seals. Cultures 8 days old at time of analysis. Results are expressed as mg.  $\text{NH}_2\text{-N}$  per 100 cc.

	Aerobic		Anaerobic	
	Total	Increases	Total	Increases
Lactic	55.8	4.8	56.2	5.2
Human	60.8	9.8	60.7	9.7
Bovine	55.6	4.6	55.8	4.8
Cheese	60.6	9.6	60.1	9.1
Control	51.0	---	51.0	---

The results given in Table XV show that the human, bovine, and lactic strains were not greatly influenced by the concentrations of oxygen tested in the above experiment. This is quite in accordance with the usual assumption that most streptococci are "facultative anaerobes". The cheese strain alone appears to attack peptone with greater avidity under aerobic conditions.

## V. Optimal Environmental Conditions for the different types as Shown by the foregoing study.

The experiments just reported offer a means of choosing the environmental conditions under which to conduct the comparison of the peptolytic activity of the different types of streptococci to be studied in Section B. The following summary presents the conditions chosen as representing optimal conditions for the different streptococci.

### Na-ion concentration zones.

Lactic and cheese strains - pH 6.5

Bovine and human strains - pH 7.5

### Temperature.

Lactic strains - approximately 30° C.

Other strains - 37° C.

### Oxygen Concentration.

Little difference was manifested between aerobic and anaerobic cultures of the different streptococci. For purpose of convenience aerobic cultures will be used in Section B.

These conditions will be maintained in the investigation reported in Section B.

## Section B.

### COMPARISON OF THE PEPTOLYTIC ACTIVITY OF DIFFERENT TYPES OF STREPTOCOCCI.

#### I. Preliminary Statements.

##### 1. Relation to preceding studies.

The preceding studies have been concerned primarily with the establishment of definite optimal environmental conditions for the peptolytic action of the different streptococci. The short summary given on the preceding page states the conditions established as optimum for the different types. The following studies are concerned with measurements of the amino and ammonia compounds which are formed by a larger number of strains of the various types when growing in peptone broth under the foregoing standardized optimum environmental conditions.

Such measurements of the relative production of these products by a number of strains of different types should furnish a certain contribution to our knowledge of differences or likenesses in metabolism between different groups of streptococci. At the same time, they offer a similar means of comparison between different members of the same type of streptococci, as now recognized in present systems of grouping these organisms.



12.

The value and worth of such comparisons is largely conditioned by the number of strains included in the tests. However, they are conditioned to an equal extent by the knowledge of other characters, and of the previous history of the strains which are studied. It is obvious that a comparison of the peptone splitting activity of the human and lactic groups of streptococci requires some assurance that the strains upon which the comparison is based possess the characters usually assigned to these groups. The number of strains included in the following tests is not large. However, it is believed that they represent a fairly typical collection of the different types. The following description of the strains is added to that furnished in the introduction.

## 2. Descriptions of groups and strains studied:

### Lactic group:

Strains from sour milk and fermented dairy products are included in this collection. In Part II of this paper, these strains are completely described, at which place evidence is presented that most of these strains would probably be included in the so-called Strep. lacticus group. Five strains (SM, S. C. W. 6) agree in all characters with Evans' characterization of this group. The remaining strains, with the possible exception of strain X, agree with the usual characterization of the lactic type. All of them reduce litmus before coagulation in litmus milk cultures, actively acidify milk, grow at low temperatures, and produce H-ion concentration more acid than pH 5.0 in glucose broth. With the exception of strain X, none of the sour milk strains are hemolytic on blood agar. Strain A represents a hemolytic sour milk strain, which, to say the least, is closely related to the typical lactic streptococci. (Strain ED, the other hemolytic sour milk strain described in Part II, was lost through a laboratory accident.)

The first mentioned five strains are termed "lactic" streptococci with apparent justification; the remaining strains are termed "non-hemolytic sour milk" and "hemolytic sour milk" strains. It is probable, however, that all of the non-hemolytic strains possess a sufficient number of characters in common to be included in the large lactic group.

### Hemolytic human group:

This group is represented by eleven strains which were furnished by Dr. O. T. Avery as a typical collection of hemolytic streptococci from human pathological conditions. These strains agree in the



following characters: exhibit final H-ion concentrations approximately pH 5.0, do not grow at 10° C., and are associated with human pathological conditions. These strains have been further described in Part II of this paper, and by Avery and O'Brien (1918), and by Dochez, Avery and Lancefield. The actual source of these strains is given in Table XVII.

#### Hemolytic mastitis or udder group:

This group is represented by eight strains which were furnished by Dr. C. E. Avery. These strains include hemolytic strains obtained from the udder and from cases of mastitis. They represent members of the "bovine" group of hemolytic streptococci. Because of the probable heterogeneous character of the "bovine" or "high-acid" group of hemolytic streptococci, our comparison is limited to its members obtained from the udders of cows. These strains differ from the preceding group in the exhibition of H-ion concentrations more acid than pH 5.0. None of these strains are able to grow at 10° C. in glucose broth.

#### Hemolytic cheese group:

This group is represented by only one strain, "Man.", which was also used as a type strain in the preceding studies. This group has not been studied to as great an extent as the preceding groups, but a complete description of the group may be found in the recent work of E. C. Avery. The description of this type strain has been given previously (Tabular Summary, Part II).

#### Non-hemolytic sauerkraut strain:

The non-hemolytic sauerkraut strain described in Part II has been included in a part of this study. It is not presented as a typical representative of any particular group, as comparatively little is known of the streptococci of fermented plant products. This strain is described quite completely in the Tabular Summary in Part II.

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## II. Comparison of Amino and of Ammonia Nitrogen Increases Exhibited by Different Members of the Lactic Group, and by Strains of Other Types of Streptococci.

It is desired to compare the increases in amino and ammonia nitrogen brought about in peptone broth by different members of the lactic group. It is further desired to compare these increases with those effected by other types of streptococci when grown under similar conditions. The attainment of these objects presents a means of showing differences and likenesses in this phase of the metabolism of different lactic streptococci, and of comparing the products accumulated in similar cultures of different types of streptococci.

The tests have been made in broths of a pH value approximating that of fresh milk. This represents a H-ion concentration some more favorable to the lactic and cheese strains than to the human and mastitis strains. Optimum temperature conditions were maintained for the various types.

### Procedure:

Infusion broth, pH 6.6, containing 0.2 per cent sodium phosphate, was inoculated with the severest lactic or non-hemolytic sour milk strains, the hemolytic sour milk strain, a representative strain of both the hemolytic human and hemolytic mastitis collections, the hemolytic cheese strain, and the non-hemolytic sauerkraut strain. The sour milk strains and the sauerkraut strain were inoculated at 31° C., the other type strains at 37° C. Amino and ammonia nitrogen determinations were made after 12 days incubation. Results are given below in Table XVI.

Table XVI.

Amino and Ammonia Nitrogen Increases Effected  
by Different Members of the Lactic Group,  
and by Strains of other types of Streptococci.

Medium: infusion broth, pH 6.6. All of the sour milk strains were incubated at 31° C.; other type strains, at 37° C. Cultures were 12 days old at the time of analysis. Results given below as mg./100cc.

				Total NH <sub>2</sub> -N	NH <sub>3</sub> -N	Increase in N NH <sub>2</sub> -N	Increase in NH <sub>3</sub> -N
Lactic strains (non-hemolytic)				61.2	16.4	4.3	7.7
SK	"	"	"	61.4	16.7	4.5	8.0
S	"	"	"	62.4	15.6	5.6	6.9
C	"	"	"	61.6	16.1	4.7	8.4
W	"	"	"	61.9	16.1	5.0	7.4
Non-hemolytic sour milk strains				60.7	---	3.8	---
IN	"	"	"	59.4	---	2.5	---
M	"	"	"	59.5	11.6	1.5	7.9
MAS	"	"	"	61.5	---	4.4	---
2	"	"	"	61.0	17.1	4.1	8.4
1	"	"	"	61.8	---	4.9	---
2	"	"	"	60.0	---	1.1	---
3	"	"	"	60.0	---	6.1	---
4	"	"	"	60.0	---	4.8	---
5	"	"	"	61.7	---	3.5	---
6	"	"	"	60.4	---	4.1	---
7	"	"	"	61.0	---	3.3	---
8	"	"	"	63.2	---	---	---
X	Hemolytic sour milk strain			62.6	15.7	6.7	7.0
Hemolytic milk strain				64.4	17.0	7.3	8.3
S62	Hemolytic milk strain			60.7	16.2	2.6	7.5
C67	Hemolytic mastitis strain			67.5	17.4	10.6	9.7
MA2	Hemolytic cheese strain			56.8	10.0	---	1.2
K	Non-hemolytic sauerkraut strain			54.9	6.7	---	---
Control				56.9	6.7	---	---

\*9-week old culture of same strain in same broth  
gave increase of 0.9 mg. NH<sub>2</sub>-N.

The results given in Table XVI show the following general relations:

The lactic and non-hemolytic sour milk strains show considerable variations in the amount of amino and ammonia compounds formed in peptone broth. The increases in amino nitrogen varied from 1.6 to 6.3 mg. per 100 cc. The increases in ammonia nitrogen varied from 2.9 to 3.0 mg. per 100 cc. Although the strain which exhibited the lowest final increase in amino nitrogen also produced the smallest increase in ammonia, there seems to be no definite relation between the increases in these two products by the various strains. Not infrequently a strain which produces a greater increase in ammonia than another strain, shows a smaller increase in amino acids.

In this medium, the hemolytic sour milk strain shows an amino nitrogen increase slightly higher than the average among the non-hemolytic strains. It does not, however, show a greater increase than that exhibited by several other sour milk strains, in this pH zone.

The hemolytic mastitis strain shows an amino nitrogen increase within the range exhibited by the lactic sour milk group. On the other hand, both the cheese and human hemolytic strains produce greater increases in amino acids than do any of the members of the lactic group. It must be remembered, of course, that the pH of this medium is more favorable to the lactics than to the mastitis and human strains.

The sauerkraut strain at the end of 12 days incubation, showed no increase in amino nitrogen. A nine week old culture of the same strain showed a small but significant increase of



0.9 mg. This finding is in accord with the discussion of the meaning of increases in amino compounds, which has been given in Section A. ("Influence of the Stage of Growth of the Culture upon Increases in Ammonia and Amino Nitrogen".)

### III. Comparison of Amino Nitrogen Increases Affected by Different Types of Streptococci.

The preceding experiment has shown that the final increases in amino nitrogen seemed to differ in the case of certain of the different types of streptococci. A greater divergence was shown to exist between the increases in amino nitrogen manifested by the different types, than between their increases in ammonia. For this reason, the present experiment was limited to comparisons of the production of amino compounds.

The primary object of the experiment is the comparison of the amino nitrogen increases brought about by different types of streptococci. The inclusion of a larger number of strains of the human and mastitis types was essential for this purpose. The study of a number of strains of these types also offered a means of comparing the amino nitrogen increases manifested by different members of the respective groups.

In this experiment, a H-ion concentration is chosen which approximates that of blood, and of the usual bacteriological media. This represents the pH zone more favorable to the human and bovine streptococci. Two strains of lactic streptococci are included as typical representatives of the lactic group. The hemolytic sour



milk strain is also included in an attempt to compare its relation to the other types, in a pH some usually more favorable to hemolytic streptococci than that used in the preceding experiment. The hemolytic cheese strain is included for comparative purposes.

Procedure: (Experiment 12).

Infusion broth, pH 7.3, containing 0.2 per cent sodium phosphate, was inoculated with each strain of the collection of human and mastitis streptococci, and with two representative lactic strains, the hemolytic sour milk strain, and the hemolytic cheese strain. After 10 days incubation at 37° C., amino nitrogen determinations were made.

Results are given in Tables XVII and XVIII.

Table XVII.

## Amino Nitrogen Increases by Streptococci from Different Sources.

Medium: Infusion broth, pH 7.3, containing 0.2 per cent sodium phosphate. Cultures incubated ten days at 37° C. before analysis. Results given as mg. /100 cc.

Strain	Source	Amino Nitrogen	
		Total	Increase
S23	Throat	59.3	7.6
S125	Throat, lobar pneumonia	61.3	9.6
S72	Throat, lobar pneumonia	61.1	9.4
S55	Sputum, broncho pneumonia	59.5	7.8
S67	Blood, broncho pneumonia	60.6	5.9
S271	Septicemia	62.7	11.0
S3	Lung autopsy, broncho pneumonia	58.3	6.6
S32	Lung autopsy, broncho pneumonia	60.9	9.2
S84	Pleural fluid	59.8	8.1
S273	Scarlet fever	61.5	9.8
S70		59.7	8.0
V1	Udder	56.4	4.7
V2	Udder	57.0	5.3
C53	Mastitis	57.4	5.7
C57	Mastitis	58.2	6.5
C59	Mastitis	55.8	4.1
C67	Mastitis	57.6	5.9
C69	Mastitis	57.4	5.7
M26	Mastitis	58.3	6.6
S	Sour milk (non-hemolytic)	58.7	4.0
G	Sour milk "	56.2	4.5
X	Sour milk (hemolytic)	59.7	8.0
MAN	Cheese (hemolytic)	60.6	8.9
Control		51.7	---

Table XVIII.

## Amino Nitrogen Increases by Different Types of Streptococci.

Results presented in Table XVII are rearranged below, with strains grouped according to types. Figures given below represent mg.  $\text{NH}_2\text{-N}/100$  cc.

Hemolytic  
Mastitis or Udder Strains

V1	4.7
V2	5.3
C53	5.7
C57	6.5
C59	4.1
C67	5.9
C69	5.7
M26	6.6

Hemolytic  
Human Strains

S23	7.6
S125	9.6
S72	9.4
S55	7.8
S67	8.9
S271	11.0
S3	6.6
S32	9.2
S84	8.1
S273	9.8
S70	8.0

Non-hemolytic Lactic  
or Sour Milk Strains.

S	4.0
G	4.5

Hemolytic  
Sour Milk Strain

X	8.0
---	-----

Hemolytic  
Cheese Strain

MAN	5.9
-----	-----

In Table XVIII, the variations in amino nitrogen production by different members of the human and the bovine hemolytic groups are seen to cover about as wide a range as that exhibited by the different non-hemolytic sour milk or lactic strains in the preceding experiment. In the pH zone of the last experiment, the mastitis collection effected approximately the same range of amino nitrogen increases as that shown by the sour milk or lactic group in the pH 6.6 zone.

In spite of the variation within the different groups, a certain divergence in the amino acid production is evident between the different types. The minimum increases effected by the human collection is no less than the maximum effected by any of the eight bovine strains studied. Of course, there is no reason to believe that a study of a larger number of strains of both types might not reveal a considerable overlapping of the range of amino acid production of the two types.

If the hemolytic sour milk strain is to be considered a member of the so-called lactic group, an example of such an overlapping of increases in amino nitrogen is presented above. However, it is also evident that the amino acid production of this strain approximates that of the representative of R. C. Avery's group of hemolytic cheese streptococci.

The variations within the different groups and the divergence in amino nitrogen increases by the different types are more evident in figure 3.

# Amino Nitrogen Increases by Different Types of Streptococci.

(values given below for Lactic strains are from Table XVI; other values, from Table XVII.)

TYPE.

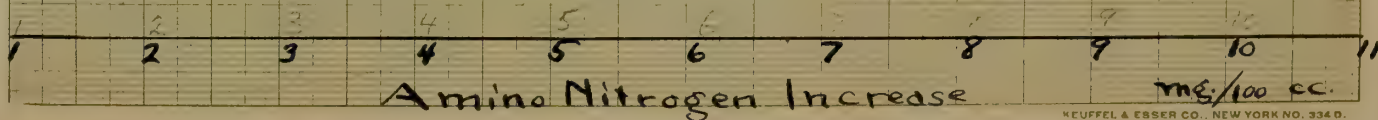
Lactic  
(non-hemolytic)

Mastitis or  
Udder  
(hemolytic)

Human  
(hemolytic)

Hemolytic Sour Milk Strain.

Hemolytic Cheese Strain.





#### IV. General Discussion of the Comparative Peptolytic Activity of Different Types of Streptococci.

##### Increases in Amino Nitrogen.

The collections of lactic or non-hemolytic sour milk strains, the hemolytic mastitis strains, and hemolytic human strains, showed considerable variation in the production of amino compounds by the different members of the various groups. In a general way, it may be said that the increases in amino nitrogen exhibited by the lactic strains studied, cover about the same range as those exhibited by the hemolytic mastitis strains. The range of amino acid production by these types is usually less than the amount produced by the representative of M. C. Avery's hemolytic cheese group. The above statements are, of course, conditioned by the number of strains studied and are limited entirely to them and to the conditions of the present experiment.

This divergence in amino acid production in peptone broth is probably of no diagnostic importance. It is, however, of peculiar interest from the standpoint of microbial physiology. Its economic importance is probably limited to its contribution to a more intimate knowledge of the metabolism of streptococci which are important in agriculture and public health.

The explanation of the apparent divergence in the extent of peptone hydrolysis, is possible only after more intimate and extended studies of the various factors and forces which are involved in the formation of amino compounds by microorganisms. Some of these have already been suggested in Section A. Among

the most important of those now recognized are the potential activity of the enzymes involved, the concentration of the enzymes, the liberation of the enzymes into the hydrolysis system itself, the stability of the enzyme activity in certain environments, and the availability of the substrate. Other forces unrecognized at the present time are probably also involved. Moreover, the relative moment of all of these forces is conditioned, and possibly to a different degree, by all of the conditions in the environment.

The question of differences in the potential activity of the enzymes of the different types of streptococci is indeed an interesting and suggestive possibility. Such questions have repeatedly been raised in connection with the relative virulence of different, but closely related microorganisms. Their solution, of course, requires the isolation of unaltered and not inactivated enzymes which is not always possible. It is always possible that there is little difference in the potential activity of similar enzymes of such closely related organisms, - and that quantitative differences observed in their products is due to the limitation of their operation by one or more of the above mentioned conditions. In consideration of the results of Avery and Gullen, the relation of virulence to the activity of the proteolytic enzymes of pneumococci would seem to be remote.

The concentration of the enzymes is closely related, but not necessarily parallel to the growth or number of the cells. It is interesting to observe that many of the human strains which give apparently delicate growth in broth culture, give much higher increases in amino nitrogen than do the seemingly more luxuriantly

growing lactic and bovine strains.

The relation of disintegration of cells and the consequent liberation of endo enzymes is also interesting, especially in consideration of the results obtained in experiment 4 (Section 4) with the human and lactic strains.

#### Increases in Ammonia Nitrogen.

The production of ammonia was found to vary to a certain extent within the lactic group. The different types of streptococci did not exhibit any marked difference in ammonia nitrogen increases in the media used. There seemed to be no definite relation between the increases in amino and in ammonia nitrogen, - neither between the different members of the lactic group nor between the strains representative of the other types.

These relations suggest that the production of ammonia is largely due to the operation of different processes than is the production of amino compounds. It was indicated in Section 4 that the formation of ammonia in peptone broth cultures is associated more strictly with the actual growth and life of streptococci than is that of amino acids.

## GENERAL SUMMARY.

## Section A.

The relative influence of environmental conditions was investigated for the purpose of establishing the optimum conditions for the peptolytic action of different types of streptococci. As an index of the successful operation of the peptolytic processes, measurements were made of the relative amounts of amino and ammonia nitrogen which were formed by the different streptococci when growing in different environments.

The results of this section of the study were used as a means of standardizing the systems to be used for the investigation in Section B. of the peptolytic action of a number of strains of the different types.

In addition to the standardization of the optimum conditions for the investigation pursued in Section B, the results of this study yielded the following summarized facts of independent interest and importance.

1. The production of amino compounds in broth cultures of streptococci seems to continue beyond the period of growth of the culture. Significant increases occur, especially with the human strain, after the cessation of growth and during the period of death of the cells.

2. A greater portion of the total increase in ammonia occurs earlier in the history of the culture than in the case of the amino acid increases. This may indicate that ammonia production is associated more strictly with the growth and active life of



streptococci than is the accumulation of amino compounds.

3. The human and bovine strains exhibit an optimum H-ion concentration zone of pH 7.0 to 8.0. The lactic and cheese strains seem to prefer a zone of pH 6.0 to 7.0. The optimum pH zone for growth of the cells agreed with the zone in which occurred the greatest amino nitrogen increase for the respective strains.

4. The ability of the cheese strain to split peptone is inhibited to a less extent by H-ion concentrations approximating pH 5.5 than is that of any of the other type strains.

5. In broth of pH 4.5, the type strains exhibit the following order of acid-tolerance: cheese, lactic, bovine, and human. The cheese strain exhibits a greater relative resistance to the disinfectant action of this concentration of the H-ion than would seem to follow from a comparison of the "fermentation limits" or final H-ion concentrations exhibited in glucose broth by the bovine and lactic strains.

6. Further examples are furnished that show that the "limiting initial H-concentrations" for different types of streptococci in plain broth, do not coincide with their "fermentation limits" in glucose broth.

7. The rate of growth of the lactic streptococcus is retarded to a less extent by low temperatures than is that of the hemolytic type strains. The same is also true of its rate of amino acid production.

8. Examples are furnished which show that higher final increases in amino nitrogen may occur at temperatures lower than that at which the greatest rate of amino acid production takes place.



9. Temperatures somewhat above the optimum may effect the final H-ion concentration of glucose broth cultures of streptococci.

10. The life processes of the cheese and bovine strains are inhibited to a less extent by a temperature of  $41^{\circ}$  than are those of the lactic and human strains. This is evident by the rate of growth, the activity of peptone attack, the final H-ion concentration, and the longevity of cultures of the different strains, when incubated at this temperature.

11. At a temperature of  $41^{\circ}$  the final amino nitrogen increases by the hemolytic human streptococcus were conditioned by the size of the inoculum. At  $37^{\circ}$ , the same limits of size of inoculum were apparently without effect upon the total production of amine acids.

12. With the exception of the cheese strain, all of the type strains proved able to attack peptone as successfully under low oxygen concentrations as under aerobic conditions.

13. The study of the influence of environmental conditions upon the different types of streptococci presents results which indicate that the lactic and cheese types are better fitted to survive the conditions extant in fresh milk, and in milk and milk products at later periods of its handling.

14. The cheese strain, by reason of its striking resistance to high acidities and to long exposures to unfavorable environments, is peculiarly adapted to the struggle for existence in the microbial balance of milk products. These relations may explain its appearance in comparatively large numbers in certain dairy products, even though it were initially present in relatively small numbers.

## Section B.

Section B is concerned with measurements of the amino and ammonia compounds which are formed by a larger number of strains of different types of streptococci when growing in peptone broth under the environmental conditions established as optimum for the different streptococci in Section A. The peptolytic action of the lactic and cheese strains were studied at  $31^{\circ}$  C. in a pH zone approximately that of fresh milk; the peptolytic action of the hemolytic human and bovine strains were studied at  $37^{\circ}$  C. in a pH zone approximately that of blood and of the usual bacteriological media.

The results obtained may be summarized as follows.

15. Broth cultures of non-hemolytic lactic, hemolytic human, and hemolytic mastitis streptococci showed considerable variation in the increases in amino and ammonia nitrogen effected by different strains of the same type.

16. The increases in amino nitrogen exhibited by the eight hemolytic mastitis strains in broth of initial pH 7.3, covered approximately the same range as that shown by seventeen non-hemolytic sour milk or lactic strains in broth of initial pH 6.6.

17. Eleven hemolytic human strains produced larger increases in amino nitrogen than did the hemolytic mastitis and non-hemolytic lactic strains.

18. With certain streptococci, increases in amino nitrogen may not be evident until after an extended period of incubation.

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